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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	9632-006-999	Total Pages 86
	First Named Inventor or Application Identifier	
	FRANCISCO	
Express Mail Label No.	EL 501 636 145 US	

APPLICATION ELEMENTS
See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

1. ☒ Fee Transmittal Form **[Total Pages 2]**
Submit an original, and a duplicate for fee processing)

2. ☒ Specification **[Total Pages 66]**
(preferred arrangement set forth below)
-Table of Contents
-Descriptive title of the Invention
-Cross Reference to Related Applications
-Statement Regarding Fed sponsored R&D
-Reference to Microfiche Appendix
-Background of the Invention
-Brief Summary of the Invention
-Brief Description of the Drawings *(if filed)*
-Detailed Description of the Invention *(including drawings, if filed)*
-Claim(s)
-Abstract of the Disclosure

3. ☒ Drawing(s) *(35 USC 113)* **[Total Sheets 6]**

4. ☒ Oath or Declaration (Unexecuted) **[Total Sheets 2]**

a. ☐ Newly executed (original or copy)
b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 5 below]
  i. ☐ DELETION OF INVENTORS(S)
  Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33 (b).

5. ☐ Incorporation By Reference *(useable if Box 4b is checked)*
The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

6. ☐ Microfiche Computer Program *(Appendix)*

7. ☒ Nucleotide and/or Amino Acid Sequence Submission
(if applicable, all necessary)

a. ☒ Computer Readable Copy

b. ☒ Paper Copy (identical to computer copy)
[Total Sheets 9]

c. ☒ Statement verifying identity of above copies
[Total Sheets 1]

ACCOMPANYING APPLICATION PARTS

8. ☐ Assignment Papers (cover sheet & document(s))

9. ☐ 37 CFR 3.73(b) Statement ☐ Power of Attorney
(when there is an assignee)

10. ☐ English Translation Document *(if applicable)*

11. ☐ Information Disclosure ☐ Copies of IDS
Statement (IDS)/PTO-1449 Citations

12. ☐ Preliminary Amendment

13. ☒ Return Receipt Postcard (MPEP 503)
(Should be specifically itemized)

14. ☐ Small Entity ☐ Statement filed in prior application,
Statement(s) Status still proper and desired

15. ☐ Certified Copy of Priority Document(s)
(if foreign priority is claimed)

16. ☐ Other:

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17. If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information: of prior application No: filed .
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ATTORNEY DOCKET NO. <u>9632-006-999</u>   Date: <u>November 28, 2000</u>

Assistant Commissioner for Patents
Box PATENT APPLICATION
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Sir:

The following utility patent application is enclosed for filing:

Applicant(s):   Francisco et al.   Executed on: Unexecuted

Title of Invention: RECOMBINANT ANTI-CD30 ANTIBODIES AND USES THEREOF

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	103	-20	83	$18.00 each	$ 1,494.00
Independent	23	-3	20	$80.00 each	$ 1,600.00
			Minimum Fee		S 710.00
			Multiple Dependency Fee .If Applicable ($270.00)		$ 270.00
			Total		$ 4,074.00
			50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern (a verified statement as to the applicant's status is attached)	-	$ 0.00
			Total Filing Fee		$ 4,074.00

☐   Priority of application no. filed on in is claimed under 35 U.S.C. § 119.
☐   The certified copy of the priority application has been filed in application no. filed
☐   Amend the specification by inserting before the first line the following sentence: This is a continuation-in-part of application no. filed .

Please charge the required fee to Pennie & Edmonds LLP Deposit Account No. 16-1150. A copy of this sheet is enclosed.

Respectfully submitted,

Adriane M. Antler   32,605
Adriane M. Antler   (Reg. No.)
PENNIE & EDMONDS LLP

Enclosure

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RECOMBINANT ANTI-CD30 ANTIBODIES AND USES THEREOF

TABLE OF CONTENTS

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RECOMBINANT ANTI-CD30 ANTIBODIES AND USES THEREOF

1. FIELD OF THE INVENTION

The present invention relates to methods and compositions for the treatment
of Hodgkin's Disease, comprising administering a protein that binds to CD30. Such
proteins include recombinant/variant forms of monoclonal antibodies AC10 and HeFi-1,
and derivatives thereof. This invention relates to a novel class of monoclonal antibodies
directed against the CD30 receptor which, in unmodified form, are capable of inhibiting the
growth of CD30-expressing Hodgkin's Disease cells.

2. BACKGROUND OF THE INVENTION

Curative chemotherapy regimens for Hodgkin's disease represent one of the
major breakthroughs in clinical oncology. Multi-agent chemotherapy regimens have
increased the cure rate to more than 80% for these patients. Nevertheless, 3% of patients
die from treatment-related causes, and for patients who do not respond to standard therapy
or relapse after first-line treatment, the only available treatment modality is high-dose
chemotherapy in combination with stem cell transplantation. This treatment is associated
with an 80% incidence of mortality, significant morbidity and a five-year survival rate of
less than 50% (See e.g., Engert, et al., 1999, Seminars in Hematology 36:282-289).

The primary cause for tumor relapse is the development of tumor cell clones
resistant to the chemotherapeutic agents. Immunotherapy represents an alterative strategy
which can potentially bypass resistance. Monoclonal antibodies for specific targeting of
malignant tumor cells has been the focus of a number of immunotherapeutic approaches.
For several malignancies, antibody-based therapeutics are now an acknowledged part of the
standard therapy. The engineered anti-CD20 antibody Rituxan®, for example, was
approved in late 1997 for the treatment of relapsed low-grade NHL.

CD30 is a 120 kilodalton membrane glycoprotein (Froese et al., 1987, J.
Immunol. 139: 2081-87) and a member of the TNF-receptor superfamily. This family
includes TNF-RI, TNF-RII, CD30, CD40, OX-40 and RANK, among others.

CD30 is a proven marker of malignant cells in Hodgkin's disease (HD) and
anaplastic large cell lymphoma (ALCL), a subset of non-Hodgkin's (NHL) lymphomas
(Dürkop et al., 1992, Cell 88:421-427). Originally identified on cultured Hodgkin's-Reed
Steinberg (H-RS) cells using the monoclonal antibody Ki-1 (Schwab et al., 1982, Nature
299:65-67), CD30 is highly expressed on the cell surface of all HD lymphomas and the
majority of ALCL, yet has very limited expression in normal tissues to small numbers of
lymphoid cells in the perifollicular areas (Josimovic-Alasevic et al., 1989, Eur. J.

Immunol. 19:157-162). Monoclonal antibodies specific for the CD30 antigen have been explored as vehicles for the delivery of cytostatic drugs, plant toxins and radioisotopes in both preclinical models and clinical studies (Engert *et al.*, 1990, Cancer Research 50:84-88; Barth *et al.*, 2000, Blood 95:3909-3914). In patients with HD, targeting of the CD30 antigen could be achieved with low doses of the anti-CD30 mAb, BerH2 (Falini *et al.*, 1992, British Journal of Haematology 82:38-45). Yet, despite successful *in vivo* targeting of the malignant tumor cells, none of the patients experienced tumor regression. In a subsequent clinical trial, a toxin (saporin) was chemically conjugated to the antibody BerH2 and all four patients demonstrated rapid and substantial reductions in tumor mass (Falini *et al.*, 1992, Lancet 339:1195-1196).

These observations underscore the validity of the CD30 receptor as a target antigen. However, all of the patients treated with the mAb-toxin conjugate developed antibodies to the toxin. One of the major limitations of immunotoxins is their inherent immunogenicity that results in the development of antibodies to the toxin molecule and neutralizes their effects (Tsutsumi *et al.*, 2000, Proc. Nat'l Acad. Sci. U.S.A. 97:8545-8553). Additionally, the liver toxicity and vascular leak syndrome associated with immunotoxins potentially limits the ability to deliver curative doses of these agents (Tsutsumi *et al.*, 2000, Proc. Nat'l Acad. Sci. U.S.A. 97:8545-8553).

2.1 CD30 MONOCLONAL ANTIBODIES

CD30 was originally identified by the monoclonal antibody Ki-1 and initially referred to as the Ki-1 antigen (Schwab *et al.*, 1982, Nature 299:65-67). This mAb was developed against Hodgkin and Reed-Sternberg (H-RS) cells, the malignant cells of Hodgkin's disease (HD). A second mAb, capable of binding a formalin resistant epitope, different from that recognized by Ki-1 was subsequently described (Schwarting *et al.*, 1989 Blood 74:1678-1689). The identification of four additional antibodies resulted in the creation of the CD30 cluster at the Third Leucocyte Typing Workshop in 1986 (McMichael, A.., ed., 1987, Leukocyte Typing III (Oxford: Oxford University Press)).

2.2 CD30 MONOCLONAL ANTIBODY-BASED THERAPEUTICS

The utility of CD30 mAbs in the diagnosis and staging of HD led to their evaluation as potential tools for immunotherapy. In patients with HD, specific targeting of the CD30 antigen was achieved with low doses (30-50 mg) of the anti-CD30 mAb BerH2 (Falini *et al.*, 1992, British Journal of Haematology 82:38-45). Despite successful targeting *in vivo* of the malignant H-RS tumor cells, none of the patients experienced tumor regressions.

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Based on these results, it was concluded that efficacy with CD30 mAb targeted immunotherapy could not be achieved with unmodified antibodies (Falini *et al.*, 1992, Lancet 339:1195-1196). In a subsequent clinical trial, treatment of four patients with refractory HD with a toxin, saporin, chemically conjugated to the mAb BerH2 demonstrated

5 rapid and substantial, although transient, reductions in tumor mass (Falini *et al.*, 1992, Lancet 339:1195-1196). In recent years, investigators have worked to refine the approaches for treating CD30-expressing neoplastic cells. Examples include the development of recombinant single chain immunotoxins (Barth *et al.*, 2000, Blood 95:3909-3914), anti-CD16/CD30 bi-specific mAbs (Renner *et al.*, 2000, Cancer Immunol. Immunother. 49:173-

10 180), and the identification of new anti-CD30 mAbs which prevent the release of CD30 molecules from the cell surface (Horn-Lohrens *et al.*, 1995, Int. J. Cancer 60:539-544). This focus has dismissed the potential of anti-CD30 mAbs with signaling activity in the treatment of Hodgkin's disease.

15 ## 2.3. IDENTIFICATION OF ANTI-CD30 MONOCLONAL ANTIBODIES WITH AGONIST ACTIVITY

In cloning and characterizing the biologic activity of the human CD30 ligand (CD30L), two mAbs, M44 and M67, were described which mimicked the activity of CD30L induced receptor crosslinking (Gruss *et al.*, 1994, Blood 83:2045-2056). In *in vitro*

20 assays, these mAbs, in immobilized form, were capable of stimulating the proliferation of activated T-cells and the Hodgkin's disease cell lines of T-cell origin, L540 and HDLM-2. In contrast, these mAbs had little effect on the Hodgkin's cell lines of B-cell origin, L428 and KM-H2 (Gruss *et al.*, 1994, Blood 83:2045-2056). In all of these assays, the binding of the CD30 receptor by the anti-CD30 mAb Ki-1 had little effect.

25 The proliferative activity of these agonist anti-CD30 mAbs on Hodgkin's cell lines suggested that anti-CD30 mAbs possessing signaling activity would not have any utility in the treatment of HD.

In contrast, the proliferation of cell lines representing CD30-expressing ALCL was strongly inhibited by the presence of immobilized M44 and M67 mAbs. This

30 inhibitory activity against ALCL cell lines was further extended to *in vivo* animal studies. The survival of SCID mice bearing ALCL tumor xenografts was significantly increased following the administration of the mAb M44. In addition, the anti-CD30 mAb HeFi-1, recognizing a similar epitope as that of M44, also prolonged survival in this animal model (Tian *et al.*, 1995, Cancer Research 55:5335-5341).

35 There is a need in the art for therapeutics with increased efficacy to treat or prevent Hodgkin's Disease, a need provided by the present invention. Clinical trials and

-3-

numerous pre-clinical evaluations have failed to demonstrate antitumor activity of a number of anti-CD30 mAbs in unmodified form against cells representative of Hodgkin's disease. Under conditions similar to those utilized by Gruss *et al.* in their evaluations of mAbs Ki-1, M44 and M67 (Gruss *et al.*, 1994, Blood 83:2045-2056), we demonstrate a class of CD30

5   mAbs which is functionally distinct from those previously described. This class of anti-CD30 mAbs is capable of inhibiting the *in vitro* growth of all Hodgkin's lines tested. Furthermore, these unmodified mAbs possess *in vivo* antitumor activity against HD tumor xenografts.

2.3.1 MONOCLONAL ANTIBODY AC10

10   The majority of murine anti-CD30 mAbs known in the art have been generated by immunization of mice with HD cell lines or purified CD30 antigen. AC10, originally termed C10 (Bowen *et al.*, 1993, J. Immunol. 151:5896-5906), is distinct in that this anti-CD30 mAb that was prepared against a human NK-like cell line, YT (Bowen *et al.*,

15   1993, J. Immunol. 151:5896-5906). Initially, the signaling activity of this mAb was evidenced by the down regulation of the cell surface expression of CD28 and CD45 molecules, the up regulation of cell surface CD25 expression and the induction of homotypic adhesion following binding of C10 to YT cells.

2.3.2 MONOCLONAL ANTIBODY HeFi-1

20   HeFi-1 is an anti-CD30 mAb which was produced by immunizing mice with the L428 Hodgkin's disease cell line (Hecht *et al.*, 1985, J. Immunol. 134:4231-4236). Co-culture of HeFi-1 with the Hodgkin's disease cell lines L428 or L540 failed to reveal any direct effect of the mAb on the viability of these cell lines. *In vitro* and *in vivo* antitumor

25   activity of HeFi-1 was described by Tian *et al* against the Karpas 299 ALCL cell line (Tian *et al.*, 1995, Cancer Research 55:5335-5341).

2.4 DIRECT ANTI-TUMOR ACTIVITY OF SIGNALING CD30 ANTIBODIES

Monoclonal antibodies represent an attractive approach to targeting specific

30   populations of cells *in vivo*. Native mAbs and their derivatives may eliminate tumor cells by a number of mechanisms including, but not limited to, complement activation, antibody dependent cellular cytotoxicity (ADCC), inhibition of cell cycle progression and induction of apoptosis (Tutt *et al.*, 1998, J. Immunol. 161:3176-3185).

As described above, mAbs to the CD30 antigen such as Ki-1 and Ber-H2

35   failed to demonstrate direct antitumor activity (Falini *et al.*, 1992, British Journal of Haematology 82:38-45; Gruss *et al.*, 1994, Blood 83:2045-2056). While some signaling

mAbs to CD30, including M44, M67 and HeFi-1, have been shown to inhibit the growth of ALCL lines *in vitro* (Gruss *et al.*, 1994, Blood 83:2045-2056) or *in vivo* (Tian *et al.*, 1995, Cancer Res. 55:5335-5341), known anti-CD30 antibodies have not been shown to be effective in inhibiting the proliferation of HD cells in culture. In fact, two signaling anti-

5   CD30 mAbs, M44 and M67, which inhibited the growth of the ALCL line Karpas-299, were shown to enhance the proliferation of T-cell-like HD lines *in vitro* while showing no effect on B-cell-like HD lines (Gruss *et al.*, 1994, Blood 83:2045-2056).

The conjugate of antibody Ki-1 with the Ricin A-chain made for a rather ineffective immunotoxin and it was concluded that this ineffectiveness was due to the rather

10   low affinity of antibody Ki-1 (Engert *et al.*, 1990, Cancer Research 50:84-88). Two other reasons may also account for the weak toxicity of Ki-1-Ricin A-chain conjugates: a) Antibody Ki-1 enhanced the release of the sCD30 from the Hodgkin-derived cell lines L428 and L540 as well as from the CD30+ non-Hodgkin's lymphoma cell line Karpas 299 (Hansen *et al.*, 1991, Immunobiol. 183:214); b) the relatively great distance of the Ki-1

15   epitope from the cell membrane is also not favorable for the construction of potent immunotoxins (Press *et al.*, 1988, J. Immunol. 141:4410-4417; May *et al.*, 1990, J. Immunol. 144:3637-3642).

At the Fourth Workshop on Leukocyte Differentiation Antigens in Vienna in February 1989, monoclonal antibodies were submitted by three different laboratories and

20   finally characterized as belonging to the CD30 group. Co-cultivation experiments by the inventors of L540 cells with various antibodies according to the state of the art, followed by the isolation of sCD30 from culture supernatant fluids, revealed that the release of the sCD30 was most strongly increased by antibody Ki-1, and weakly enhanced by the antibody HeFi-1, whilst being more strongly inhibited by the antibody Ber-H2. However,

25   the antibody Ber-H2 also labels a subpopulation of plasma cells (Schwarting *et al.*, 1988, Blood 74:1678-1689) and G. Pallesen (G. Pallesen, 1990, Histopathology 16:409-413) describes, on page 411, that Ber-H2 is cross-reacting with an epitope of an unrelated antigen which is altered by formaldehyde.

Citation or identification of any reference herein shall not be construed as an

30   admission that such reference is available as prior art to the present invention.

3.   SUMMARY OF THE INVENTION

The present invention is based on the surprising discovery of a novel activity associated with a certain class of anti-CD30 antibodies, said class comprising AC10 and

35   HeFi-1, namely their ability to inhibit the growth of both T-cell-like and B-cell-like Hodgkin's Disease (HD) cells.

The invention provides proteins that compete for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and exert a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line. The invention further provides antibodies that immunospecifically bind CD30 and exert a cytostatic or cytotoxic effect on a Hodgkin's

5   Disease cell line.

The invention further provides a method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject, in an amount effective for said treatment or prevention, an antibody that immunospecifically binds CD30 and exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line; and a

10   pharmaceutically acceptable carrier. The invention provides a method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, which amount is effective for the treatment or prevention of Hodgkin's

15   Disease. In one embodiment, a protein of the invention is conjugated to a cytotoxic molecule. In another embodiment, a protein of the invention is a fusion protein comprising the amino acid sequence of a second protein such as bryodin or a pro-drug converting enzyme. The proteins of the invention, including conjugates and fusion proteins, can be used in conjunction with radiation therapy, chemotherapy, hormonal therapy and/or

20   immunotherapy.

In determining the cytostatic effect of the proteins of the invention on Hodgkin's Disease cell lines, a culture of the Hodgkin's Disease cell line is contacted with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours; exposed to 0.5 μCi of ^{3}H-thymidine during the

25   final 8 hours of said 72-hour period; and the incorporation of ^{3}H-thymidine into cells of the culture, is measured. The protein has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein. Suitable Hodgkin's Disease cell lines to

30   determine the cytostatic or cytotoxic effects of the proteins of the invention are L428, L450, HDLM2 or KM-H2.

Wherein the protein of the invention is an antibody, the antibody is a monoclonal antibody, preferably a recombinant antibody, and most preferably is human, humanized, or chimeric.

35   The invention further provides isolated nucleic acids encoding a protein that competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and exerts a

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cytostatic or cytotoxic effect on a Hodgkin's Disease cell line. The invention further provides methods of isolating nucleic acids encoding antibodies that immunospecifically bind CD30 and exert a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line.

The invention further provides a method of producing a protein comprising
5 growing a cell containing a recombinant nucleotide sequence encoding a protein, which protein competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1 and exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, such that the protein is expressed by the cell; and recovering the expressed protein.

The invention yet further provides a method for identifying an anti-CD30
10 antibody useful for the treatment or prevention of Hodgkin's Disease, comprising determining whether the anti-CD30 antibody exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line by contacting a culture of the Hodgkin's Disease cell line with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours; exposing the culture to 0.5 μCi of ^{3}H-thymidine
15 during the final 8 hours of said 72-hour period; and measuring the incorporation of ^{3}H-thymidine into cells of the culture. The anti-CD30 antibody has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line and is useful for the treatment or prevention of Hodgkin's Disease if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same
20 conditions but not contacted with the anti-CD30 antibody.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Growth inhibition of Hodgkin's disease cell lines: Hodgkin's
25 disease cell lines HDLM-2, L540, L428 and KM-H2 were cultured at 5x10^4 cells/well in the presence or absence of 10 μg/ml of immobilized AC10. Ki-1 was used as a control in these assays. Proliferation was measured by ^{3}H-thymidine incorporation following 72 hours of culture.

FIG. 2. Growth inhibition of Hodgkin's disease cell lines: Hodgkin's
30 disease cell lines HDLM-2, L540, L428 and KM-H2 were cultured at 5x10^3 cells/well in the presence or absence of 10 μg/ml of immobilized AC10. Ki-1 was used as a control in these assays. Proliferation was measured by ^{3}H-thymidine incorporation following 72 hours of culture.

35

FIG. 3. Growth inhibition of Hodgkin's disease cell lines: Hodgkin's disease cell lines HDLM-2, L540, L428 and KM-H2 were cultured at 5×10^4 cells/well in the presence or absence of 0.1 μg/ml AC10 or HeFi- 1 that had been cross-linked by the addition of 20 μg/ml polyclonal goat anti-mouse IgG antibodies. Proliferation was measured by ^{3}H-thymidine incorporation following 72 hours of culture.

FIG. 4. Growth inhibition of Hodgkin's disease cell lines: Hodgkin's disease cell lines HDLM-2, L540, L428 and KM-H2 were cultured at 5×10^3 cells/well in the presence or absence of 0.1 μg/ml AC10 or HeFi-1 that had been cross-linked by the addition of 20 μg/ml polyclonal goat anti-mouse IgG antibodies. Proliferation was measured by ^{3}H-thymidine incorporation following 72 hours of culture.

FIG. 5. Antitumor activity of AC10 (circles) and HeFi-1 (squares) in disseminated (A) and subcutaneous (B) L540cy Hodgkin's disease xenografts. A) Mice were implanted with 1×10^7 cells through the tail vein on day 0 and received intraperitoneal injections of antibody at 1 mg/kg/injection using an administration schedule of q2dx10. B) Mice were implanted subcutaneously with 2×10^7 L540cy cells. When tumors were palpable mice were treated with intraperitoneal injections of AC10 or HeFi-1 at 2 mg/kg/injection q2dx10. In both experiments untreated mice (X) received no therapy.

FIG. 6. Antitumor activity of chimeric AC10 (cAC10) in subcutaneous L540cy Hodgkin's disease xenografts. SCID mice were implanted subcutaneously with L540cy cells and when the tumors reached an average size of >150 mm^3 mice were either left untreated (X) or treated with cAC10 (□) at 2 mg/kg twice per week for 5 injections.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to proteins that bind to CD30 and exert a cytostatic or cytotoxic effect on HD cells. The invention further relates to proteins that compete with AC10 or HeFi-1 for binding to CD30 and exert a cytostatic or cytotoxic effect on HD cells. In one embodiment, the protein is an antibody. In a preferred mode of the embodiment, the antibody is AC10 or HeFi-1, most preferably a humanized or chimeric AC10 or HeFi-1.

The invention further relates to proteins encoded by and nucleotide sequences of AC10 and HeFi-1 genes. The invention further relates to fragments and other derivatives and analogs of such AC10 and HeFi-1 proteins. Nucleic acids encoding such

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fragments or derivatives are also within the scope of the invention. Production of the foregoing proteins, e.g., by recombinant methods, is provided.

The invention also relates to AC10 and HeFi-1 proteins and derivatives including fusion/chimeric proteins which are functionally active, i.e., which are capable of displaying binding to CD30 and exerting a cytostatic or cytotoxic effect on HD cells.

Antibodies to CD30 encompassed by the invention include human, chimeric or humanized antibodies, and such antibodies conjugated to cytotoxic agents such chemotherapeutic drugs.

The invention further relates to methods of treating or preventing HD comprising administering a composition comprising a protein or nucleic acid of the invention alone or in combination with a cytotoxic agent, including but not limited to a chemotherapeutic drug.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5.1   PROTEINS OF THE INVENTION

The present invention encompasses proteins, including but not limited to antibodies, that bind to CD30 and exert cytostatic and/or cytotoxic effects on HD cells. The invention further relates to proteins that compete with AC10 or HeFi-1 for binding to CD30 and exert a cytostatic or cytotoxic effect on HD cells.

The present invention further encompasses proteins comprising, or alternatively consisting of, a CDR of HeFi-1 (SEQ ID NO:20, SEQ ID NO:22; SEQ ID NO:24; SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32) or AC10 (SEQ ID NO:4; SEQ ID NO:6; SEQ ID NO:8; SEQ ID NO:12; SEQ ID NO:14; or SEQ ID NO:16).

The present invention further encompasses proteins comprising, or alternatively consisting of, a variable region of HeFi-1 (SEQ ID NO:18 or SEQ ID NO:26) or AC10 (SEQ ID NO:2 or SEQ ID NO:10). A table indicating the region of AC10 or HeFi-1 to which each SEQ ID NO corresponds to is provided below:

Table 1

MOLECULE	NUCLEOTIDE OR AMINO ACID	SEQ ID NO
AC10 Heavy Chain Variable Region	Nucleotide	1
AC10 Heavy Chain Variable Region	Amino Acid	2
AC10 Heavy Chain-CDR1(H1)	Nucleotide	3
AC 10 Heavy Chain-CDR1(H1)	Amino Acid	4

-9-

MOLECULE	NUCLEOTIDE OR AMINO ACID	SEQ ID NO
AC 10 Heavy Chain-CDR2(H2)	Nucleotide	5
AC 10 Heavy Chain-CDR2(H2)	Amino Acid	6
AC 10 Heavy Chain-CDR3(H3)	Nucleotide	7
AC 10 Heavy Chain-CDR3(H3)	Amino Acid	8
AC 10 Light Chain Variable Region	Nucleotide	9
AC 10 Light Chain Variable Region	Amino Acid	10
AC 10 Light Chain-CDR1(L1)	Nucleotide	11
AC 10 Light Chain-CDR1(L1)	Amino Acid	12
AC 10 Light Chain-CDR2(L2)	Nucleotide	13
AC 10 Light Chain-CDR2(L2)	Amino Acid	14
AC 10 Light Chain-CDR3(L3)	Nucleotide	15
AC 10 Light Chain-CDR3(L3)	Amino Acid	16
HeFi-1 Heavy Chain Variable Region	Nucleotide	17
HeFi-1 Heavy Chain Variable Region	Amino Acid	18
HeFi-1 Heavy Chain-CDR1(H1)	Nucleotide	19
HeFi-1 Heavy Chain-CDR1(H1)	Amino Acid	20
HeFi-1 Heavy Chain-CDR2(H2)	Nucleotide	21
HeFi-1 Heavy Chain-CDR2(H2)	Amino Acid	22
HeFi-1 Heavy Chain-CDR3(H3)	Nucleotide	23
HeFi-1 Heavy Chain-CDR3(H3)	Amino Acid	24
HeFi-1 Light Chain Variable Region	Nucleotide	25
HeFi-1 Light Chain Variable Region	Amino Acid	26
HeFi-1 Light Chain-CDR1(L1)	Nucleotide	27
HeFi-1 Light Chain-CDR1(L1)	Amino Acid	28
HeFi-1 Light Chain-CDR2(L2)	Nucleotide	29
HeFi-1 Light Chain-CDR2(L2)	Amino Acid	30
HeFi-1 Light Chain-CDR3(L3)	Nucleotide	31
HeFi-1 Light Chain-CDR3(L3)	Amino Acid	32

The present invention further comprises functional derivatives or analogs of AC10 and HeFi-1. As used herein, the term "functional" in the context of a peptide or

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protein of the invention indicates that the peptide or protein is 1) capable of binding to CD30 and 2) exerts a cytostatic and/or cytotoxic effect on HD cells.

Generally, antibodies of the invention immunospecifically bind CD30 and exert cytostatic and cytotoxic effects on malignant cells in HD. Antibodies of the invention are preferably monoclonal, and may be multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, and CD30 binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that immunospecifically binds CD30. The immunoglobulin molecules of the invention can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

In certain embodiments of the invention, the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and F(ab')$_2$, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V$_L$ or V$_H$ domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, CH3 and CL domains. Also included in the invention are antigen-binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, CH3 and CL domains. Preferably, the antibodies are human, murine (*e.g.*, mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B cells, or from animals transgenic for one or more human immunoglobulin, as described *infra* and, for example in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

The antibodies of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of CD30 or may be specific for both CD30 as well as for a heterologous protein. *See, e.g.*, PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, *et al.*, 1991, J. Immunol. 147:60-69; U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny *et al.*, 1992, J. Immunol. 148:1547-1553.

Antibodies of the present invention may be described or specified in terms of the particular CDRs they comprise. In certain embodiments antibodies of the invention comprise one or more CDRs of AC10 and/or HeFi-1. The invention encompasses an

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antibody or derivative thereof comprising a heavy or light chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs are from monoclonal antibody AC10 or HeFi-1, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody AC10 or HeFi-1, respectively, and in which said antibody or derivative thereof immunospecifically binds CD30.

In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a heavy chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:4, 6, or 8 and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody AC10, and in which said antibody or derivative thereof immunospecifically binds CD30.

In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a heavy chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:20, 22 or 24 and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody HeFi-1, and in which said antibody or derivative thereof immunospecifically binds CD30.

In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a light chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:12, 14 or 16, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody AC10, and in which said antibody or derivative thereof immunospecifically binds CD30.

In a specific embodiment, the invention encompasses an antibody or derivative thereof comprising a light chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:28, 30, or 32, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody HeFi-1, and in which said antibody or derivative thereof immunospecifically binds CD30.

Additionally, antibodies of the present invention may also be described or specified in terms of their primary structures. Antibodies having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% and most preferably at least 98% identity (as calculated using methods known in the art and described herein) to the variable regions and AC10 or HeFi-1 are also included in the present invention. Antibodies of the present invention may also be

described or specified in terms of their binding affinity to CD30. Preferred binding affinities include those with a dissociation constant or Kd less than 5×10^{-2} M, 10^{-2} M, 5×10^{-3} M, 10^{-3} M, 5×10^{-4} M, 10^{-4} M, 5×10^{-5} M, 10^{-5} M, 5×10^{-6} M, 10^{-6} M, 5×10^{-7} M, 10^{-7} M, 5×10^{-8} M, 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, $5 \times ^{-13}$ M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, or 10^{-15} M.

The antibodies of the invention include derivatives that are modified, *i.e,* by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from binding to CD30 or from exerting a cytostatic or cytotoxic effect on HD cells. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, *etc.* Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies of the present invention may be generated by any suitable method known in the art. Polyclonal antibodies to CD30 can be produced by various procedures well known in the art. For example, CD30 can be administered to various host animals including, but not limited to, rabbits, mice, rats, *etc.* to induce the production of sera containing polyclonal antibodies specific for the protein. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed., 1988); Hammerling, *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody"

refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with CD30 or a cell expressing CD30 or a fragment or derivative thereof. Once an immune response is detected, e.g., antibodies specific for CD30 are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding CD30. Ascites fluid, which generally contains high levels of antibodies, can be generated by injecting mice with positive hybridoma clones.

Accordingly, the present invention provides methods of generating monoclonal antibodies as well as antibodies produced by the method comprising culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from a mouse immunized with an antigen of the invention with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind to CD30.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). $F(ab')_2$ fragments contain the variable region, the light chain constant region and the CH 1 domain of the heavy chain.

For example, the antibodies of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the nucleic acid sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the nucleic acid sequences encoding them. In particular, DNA sequences encoding V_H and V_L domains are amplified from animal cDNA libraries (e.g., human or murine cDNA libraries of lymphoid tissues). The DNA encoding the V_H and V_L domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector (e.g., p CANTAB 6 or pComb 3 HSS).

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The vector is electroporated in *E. coli* and the *E. coli* is infected with helper phage. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage expressing an

5   antigen binding domain that binds to CD30 or an AC10 or HeFi- binding portion thereof can be selected or identified with antigen *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman *et al.*, 1995, J. Immunol. Methods 182:41-50; Ames *et al.*, 1995, J. Immunol. Methods 184:177-

10   186; Kettleborough *et al.*, 1994, Eur. J. Immunol. 24:952-958; Persic *et al.*, 1997, Gene 187:9-18; Burton *et al.*, 1994, Advances in Immunology, 191-280; PCT Application No. PCT/GB91/01 134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/1 1236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908;

15   5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

     As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed

20   in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and F(ab')$_2$ fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax *et al.*, BioTechniques 1992, 12(6):864-869; and Sawai *et al.*, 1995, AJRI 34:26-34; and Better *et al.*, 1988, Science

25   240:1041-1043 (said references incorporated by reference in their entireties).

     Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston *et al.*, 1991, Methods in Enzymology 203:46-88 ; Shu *et al.*, 1993, PNAS 90:7995-7999; and Skerra *et al.*, 1988, Science 240:1038-1040 . For some uses, including *in vivo* use of

30   antibodies in humans and *in vitro* proliferation or cytotoxicity assays, it is preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are

35   known in the art. *See e.g.*, Morrison, Science, 1985, 229:1202 ; Oi *et al.*, 1986, BioTechniques 4:214; Gillies *et al.*, 1989, J. Immunol. Methods 125:191-202; U.S. Patent

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Nos. 5,807,715; 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibody that binds the desired antigen having one or more CDRs from the non-human species and framework and constant regions from a human immunoglobulin molecule.

5   Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, e.g., by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify

10 unusual framework residues at particular positions. (See, e.g., Queen et al., U.S. Patent No. 5,585,089; Riechmann et al., , 1988, Nature $\underline{332}$:323 , which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 9 1/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing

15 (EP 592,106; EP 519,596; Padlan, Molecular Immunology, 1991, $\underline{28}$(4/5):489-498; Studnicka et al., 1994, Protein Engineering $\underline{7}$(6):805-814; Roguska. et al., 1994, PNAS $\underline{91}$:969-973), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Human antibodies can be made by a variety of methods

20 known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

25 Human antibodies can also be produced using transgenic mice which express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with

30 the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion

35 with a selected antigen, e.g., all or a portion of CD30. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using

conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this

5   technology for producing human antibodies, *see*, Lonberg and Huszar, 1995, Int. Rev. Immunol. 13:65-93. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see*, *e.g.*, PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425;

10 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Genpharm (San Jose, CA) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

15   Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.*, 1994, Bio/technology 12:899-903).

20   Further, antibodies to CD30 can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" proteins of the invention using techniques well known to those skilled in the art. (*See*, *e.g.*, Greenspan & Bona, 1989, FASEB J. 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). Fab fragments of such anti-idiotypes can be used in therapeutic regimens to elicit an individual's own immune response against

25 CD30 and HD cells.

As alluded to above, proteins that are therapeutically or prophylactically useful against HD need not be antibodies. Accordingly, proteins of the invention may comprise one or more CDRs from an antibody that binds to CD30 and exerts a cytotoxic and/or cytostatic effect on HD cells. Preferably, a protein of the invention is a multimer,

30 most preferably a dimer.

The invention also provides proteins, including but not limited to antibodies, that competitively inhibit binding of AC10 or HeFi-1 to CD30 as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the protein competitively

35 inhibits binding of AC10 or HeFi-1 to CD30 by at least 50%, more preferably at least 60%, yet more preferably at least 70%, and most preferably at least 75%. In other embodiments,

the protein competitively inhibits binding of AC10 or HeFi-1 to CD30 by at least 80%, at least 85%, at least 90%, or at least 95%.

As discussed in more detail below, the proteins of the present invention may be used either alone or in combination with other compositions in the prevention or
5 treatment of HD. The proteins may further be recombinantly fused to a heterologous protein at the N- or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to cytotoxic agents, proteins or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as chemotherapeutics or toxins, or comprise a radionuclide for use as a radio-
10 therapeutic. *See, e.g.,* PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

Proteins of the invention may be produced recombinantly by fusing the coding region of one or more of the CDRs of an antibody of the invention in frame with a sequence coding for a heterologous protein. The heterologous protein may provide one or
15 more of the following characteristics: added therapeutic benefits; promote stable expression of the protein of the invention; provide a means of facilitating high yield recombinant expression of the protein of the invention; or provide a multimerization domain.

In addition to proteins comprising one or more CDRs of an antibody of the invention, proteins of the invention may be identified using any method suitable for
20 screening for protein-protein interactions. Initially, proteins are identified that bind to CD30, then their ability to exert a cytostatic or cytotoxic effect on HD cells can be determined. Among the traditional methods which can be employed are "interaction cloning" techniques which entail probing expression libraries with labeled CD30 in a manner similar to the technique of antibody probing of λgt11 libraries, *supra*. By way of
25 example and not limitation, this can be achieved as follows: a cDNA clone encoding CD30 (or an AC10 or HeFi-1 binding domain thereof) is modified at the terminus by inserting the phosphorylation site for the heart muscle kinase (HMK) (Blanar & Rutter, 1992, Science 256:1014-1018). The recombinant protein is expressed in *E. coli* and purified on a GDP-affinity column to homogeneity (Edery *et al.*, 1988, Gene 74:517-525) and labeled
30 using γ^{32}P-ATP and bovine heart muscle kinase (Sigma) to a specific activity of 1x10^8 cpm/μg, and used to screen a human placenta λgt11 cDNA library in a "far-Western assay" (Blanar & Rutter, 1992, Science 256:1014-1018). Plaques which interact with the CD30 probe are isolated. The cDNA inserts of positive λ plaques are released and subcloned into a vector suitable for sequencing, such as pBluescript KS (Stratagene).
35 One method which detects protein interactions *in vivo*, the two-hybrid system, is described in detail for illustration purposes only and not by way of limitation.

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One version of this system has been described (Chien *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA).

Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one consists of the DNA-binding domain of a transcription activator protein fused to CD30, and the other consists of the activator protein's activation domain fused to an unknown protein that is encoded by a cDNA which has been recombined into this plasmid as part of a cDNA library. The plasmids are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (*e.g.*, lacZ) whose regulatory region contains the transcription activator's binding sites. Either hybrid protein alone cannot activate transcription of the reporter gene, the DNA-binding domain hybrid cannot because it does not provide activation function, and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology can be used to screen activation domain libraries for proteins that interact with CD30, which in this context is a "bait" gene product. Total genomic or cDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of a CD30 coding region (for example, a nucleotide sequence which codes for a domain of CD30 known to interact with HeFi-1 or AC10) fused to the DNA-binding domain are co-transformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, and not by way of limitation, the CD30 coding region can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

Once a CD30-binding protein is identified, its ability (alone or when multimerized or fused to a dimerization or multimerization domain) to elicit a cytostatic or cytotoxic effect on HD cells is determined by contacting a culture of an HD cell line, such as L428, L450, HDLM2 or KM-H2, with the protein. Culture conditions are most preferably about 5,000 cells in a culture area of about 0.33 cm^2, and the contacting period being approximately 72 hours. The culture is then exposed to 0.5 µCi of ^{3}H-thymidine during the final 8 hours of the 72-hour period and the incorporation of ^{3}H-thymidine into cells of the culture is measured. The protein has a cytostatic or cytotoxic effect on the HD cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to

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cells of the same cell line cultured under the same conditions but not contacted with the protein.

Without limitation as to mechanism of action, a protein of the invention preferably has more than one CD30-binding site and therefore a capacity to cross link CD30 molecules. Proteins which bind to CD30 or compete for binding to CD30 with AC10 or HeFi-1 can acquire the ability to induce cytostatic or cytotoxic effects on HD cells if dimerized or multimerized. Wherein the CD30-binding protein is a monomeric protein, it can be expressed in tandem, thereby resulting in a protein with multiple CD30 binding sites. The CD30-binding sites can be separated by a flexible linker region. In another embodiment, the CD30-binding proteins can be chemically cross-linked, for example using gluteraldehyde, prior to administration. In a preferred embodiment, the CD30-binding region is fused with a heterologous protein, wherein the heterologous protein comprises a dimerization and multimerization domain. Prior to administration of the protein of the invention to a subject for the purpose of treating or preventing HD, such a protein is subjected to conditions that allows formation of a homodimer or heterodimer. A heterodimer, as used herein, may comprise identical dimerization domains but different CD30-binding regions, identical CD30-binding regions but different dimerization domains, or different CD30-binding regions and dimerization domains.

Particularly preferred dimerization domains are those that originate from transcription factors.

In one embodiment, the dimerization domain is that of a basic region leucine zipper ("bZIP"). bZIP proteins characteristically possess two domains--a leucine zipper structural domain and a basic domain that is rich in basic amino acids, separated by a "fork" domain (C. Vinson et al., 1989, Science, 246:911-916). Two bZIP proteins dimerize by forming a coiled coil region in which the leucine zipper domains dimerize. Accordingly, these coiled coil regions may be used as fusion partners for the proteins and the invention.

Particularly useful leucine zipper domain are those of the yeast transcription factor GCN4, the mammalian transcription factor CCAAT/enhancer-binding protein C/EBP, and the nuclear transform in oncogene products, Fos and Jun (see Landschultz et al., 1988, Science 240:1759-1764; Baxevanis and Vinson, 1993, Curr. Op. Gen. Devel., 3:278-285; and O'Shea et al., 1989, Science, 243:538-542).

In another embodiment, the dimerization domain is that of a basic-region helix-loop-helix ("bHLH") protein (Murre et al, 1989, Cell, 56:777-783). bHLH proteins are also composed of discrete domains, the structure of which allows them to recognize and interact with specific sequences of DNA. The helix-loop-helix region promotes dimerization through its amphipathic helices in a fashion analogous to that of the leucine

zipper region of the bZIP proteins (Davis *et al.*, 1990 Cell, 60:733-746; Voronova and Baltimore, 1990 Proc. Natl. Acad. Sci. USA, 87:4722-4726). Particularly useful hHLH proteins are myc, max, and mac.

Heterodimers are known to form between Fos and Jun (Bohmann *et al.*,
5 1987, Science, 238:1386-1392), among members of the ATF/CREB family (Hai *et al.*,1989, Genes Dev., 3:2083-2090), among members of the C/EBP family (Cao *et al.*, 1991, Genes Dev., 5:1538-1552; Williams *et al.*, 1991, Genes Dev., 5:1553-1567; and Roman *et al.*, 1990, Genes Dev., 4:1404-1415), and between members of the ATF/CREB and Fos/Jun families Hai and Curran, 1991, Proc. Natl. Acad. Sci. USA, 88:3720-3724). Therefore,
10 when a protein of the invention is administered to a subject as a heterodimer comprising different dimerization domains, any combination of the foregoing may be used.

5.2   BINDING ASSAYS

As described above, the proteins, including antibodies, of the invention bind
15 to CD30 and exert a cytostatic or cytotoxic effect on HD cells. Methods of demonstrating the ability of a protein of the invention to bind to CD30 are described herein.

The antibodies of the invention may be assayed for immunospecific binding to CD30 by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such
20 as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (*see, e.g.*, Ausubel *et.*
25 *al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium
30 deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (*e.g.*, EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody to the cell lysate, incubating for a period of time (*e.g.*, 1-4 hours) at 40° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 40° C, washing the beads in lysis buffer
35 and resuspending the beads in SDS/sample buffer. The ability of the antibody to immunoprecipitate CD30 can be assessed by, *e.g.*, Western blot analysis. One of skill in the

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art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to CD30 and decrease the background (*e.g.*, pre-clearing the cell lysate with sepharose beads). For further discussion regarding immunoprecipitation protocols *see, e.g.*, Ausubel *et al., eds.*, 1994, Current Protocols in Molecular Biology, Vol.

5 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (*e.g.*, 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, incubating the

10 membrane in blocking solution (*e.g.*, PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (*e.g.*, PBS-Tween 20), blocking the membrane with primary antibody (*i.e.*, the putative anti-CD30 antibody) diluted in blocking buffer, washing the membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.*, an anti-human antibody) conjugated to an enzyme

15 substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) or radioactive molecule (*e.g.*, ^{32}P or ^{125}I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the secondary antibody. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding Western blot protocols

20 *see, e.g.*, Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen (*i.e.*, CD30), coating the well of a 96 well microtiter plate with the CD30, adding the antibody conjugated to a detectable compound such as an enzyme (*e.g.*, horseradish peroxidase or alkaline phosphatase) to the

25 well and incubating for a period of time, and detecting the presence of the antibody. In ELISAs the antibody does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a

30 detectable compound may be added following the addition of CD30 protein to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs *see, e.g.*, Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

35 The binding affinity of an antibody to CD30 and the off-rate of an antibody CD30 interaction can be determined by competitive binding assays. One example of a

competitive binding assay is a radioimmunoassay comprising the incubation of labeled CD30 (*e.g.*, ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled CD30, and the detection of the antibody bound to the labeled CD30. The affinity of the antibody for CD30 and the binding off-rates can then be determined from the data by

5   Scatchard plot analysis. Competition with a second antibody (such as AC10 or HeFi-1) can also be determined using radioimmunoassays. In this case, CD30 is incubated with the antibody of interest conjugated to a labeled compound (*e.g.*, ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody.

Proteins of the invention may also be assayed for their ability to bind to

10   CD30 by a standard assay known in the art. Such assays include far Westerns and the yeast two hybrid system. These assays are described in Section 5.2, *supra*. Another variation on the far Western technique described above entails measuring the ability of a labeled candidate protein to bind to CD30 in a Western blot. In one non-limiting example of a far Western blot, CD30 or the fragment thereof of interest is expressed as a fusion protein

15   further comprising glutathione-S-transferase (GST) and a protein serine/threonine kinase recognition site (such as a cAMP-dependent kinase recognition site). The fusion protein is purified on glutathione-Sepharose beads (Pharmacia Biotech) and labeled with bovine heart kinase (Sigma) and 100 μCi of ^{32}P-ATP (Amersham). The test protein(s) of interest are separated by SDS-PAGE and blotted to a nitrocellulose membrane, then incubated with the

20   labeled CD30. Thereafter, the membrane is washed and the radioactivity quantitated. Conversely, the protein of interest can be labeled by the same method and used to probe a nitrocellulose membrane onto which CD30 has been blotted.

5.3   ASSAYS FOR CYTOTOXIC AND CYTOSTATIC ACTIVITIES

25   By definition, a protein of the invention must exert a cytostatic or cytotoxic effect on a cell of HD. Suitable HD cell lines for this purpose include L428, L450, HDLM2 and KM-H2 (all of which are available from the German Collection of Microorganisms and Cell Cultures (DMSZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)).

30   Many methods of determining whether a protein exerts a cytostatic or cytotoxic effect on a cell are known to those of skill in the art, and can be used to elucidate whether a particular protein is a protein of the invention. Illustrative examples of such methods are described below.

Wherein a protein that binds to CD30 does not exert a cytostatic or cytotoxic

35   effect on HD cells, the protein can be multimerized according to the methods described in

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Section 5.1, *supra,* and the multimer assayed for its ability to exert a cytostatic or cytotoxic effect on HD cells.

Once a protein is identified that both (i) binds to CD30 and (ii) exerts a cytostatic or cytotoxic effect on HD cells, its therapeutic value is validated in an animal model, as described in Section 6, *infra*.

In a preferred embodiment, determining whether a protein exerts a cytostatic or cytotoxic effect on a HD cell line can be made by contacting a 5,000 cell-culture of the HD cell line in a culture area of about 0.33 cm^2 with the protein for a period of 72 hours. During the last 8 hours of the 72-hour period, the culture is exposed to 0.5 μCi of ^{3}H-thymidine. The incorporation of ^{3}H-thymidine into cells of the culture is then measured. The protein has a cytostatic or cytotoxic effect on the HD cell line and is useful for the treatment or prevention of HD if the cells of the culture contacted with the protein have reduced ^{3}H-thymidine incorporation compared to cells of the same HD cell line cultured under the same conditions but not contacted with the anti-CD30 antibody.

There are many cytotoxicity assays known to those of skill in the art. Some of these assays measure necrosis, while others measure apoptosis (programmed cell death). Necrosis is accompanied by increased permeability of the plasma membrane; the cells swell and the plasma membrane ruptures within minutes. On the other hand, apoptosis is characterized by membrane blebbing, condensation of cytoplasm and the activation of endogenous endonucleases. Only one of these effects on HD cells is sufficient to show that a CD30-binding protein is useful in the treatment or prevention of HD as an alternative to the assays measuring cytostatic or cytotoxic effects described above.

In one embodiment, necrosis measured by the ability or inability of a cell to take up a dye such as neutral red, trypan blue, or ALAMAR™ blue (Page *et al.,* 1993, Intl. J. of Oncology 3:473-476). In such an assay, the cells are incubated in media containing the dye, the cells are washed, and the remaining dye, reflecting cellular uptake of the dye, is measured spectrophotometrically.

In another embodiment, the dye is sulforhodamine B (SRB), whose binding to proteins can be used as a measure of cytotoxicity (Skehan et al., 1990, J. Nat'l Cancer Inst. 82:1107-12).

In yet another embodiment, a tetrazolium salt, such as MTT, is used in a quantitative colorimetric assay for mammalian cell survival and proliferation by detecting living, but not dead, cells (*see, e.g.,* Mosmann, 1983, J. Immunol. Methods 65:55-63).

In yet another embodiment, apoptotic cells are measured in both the attached and "floating" compartments of the cultures. Both compartments are collected by removing the supernatant, trypsinizing the attached cells, and combining both preparations following

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a centrifugation wash step (10 minutes, 2000 rpm). The protocol for treating tumor cell cultures with sulindac and related compounds to obtain a significant amount of apoptosis has been described in the literature (*see, e.g.*, Piazza *et al.*, 1995, Cancer Research 55:3110-16). Features of this method include collecting both floating and attached cells,

5   identification of the optimal treatment times and dose range for observing apoptosis, and identification of optimal cell culture conditions.

In yet another embodiment, apoptosis is quantitated by measuring DNA fragmentation. Commercial photometric methods for the quantitative *in vitro* determination of DNA fragmentation are available. Examples of such assays, including TUNEL (which

10   detects incorporation of labeled nucleotides in fragmented DNA) and ELISA-based assays, are described in Biochemica, 1999, no. 2, pp. 34-37 (Roche Molecular Biochemicals).

In yet another embodiment, apoptosis can be observed morphologically. Following treatment with a test protein or nucleic acid, cultures can be assayed for apoptosis and necrosis by fluorescent microscopy following labeling with acridine orange

15   and ethidium bromide. The method for measuring apoptotic cell number has previously been described by Duke & Cohen, 1992, Current Protocols In Immunology, Coligan *et al.*, eds., 3.17.1-3.17.16. In another mode of the embodiment, cells can be labeled with the DNA dye propidium iodide, and the cells observed for morphological changes such as chromatin condensation and margination along the inner nuclear membrane, cytoplasmic

20   condensation, increased membrane blebbing and cellular shrinkage.

5.4   NUCLEIC ACIDS OF THE INVENTION

The invention further provides nucleic acids comprising a nucleotide sequence encoding a protein, including but not limited to, a protein of the invention and

25   fragments thereof. Nucleic acids of the invention preferably encode one or more CDRs of antibodies that bind to CD30 and exert cytotoxic or cytostatic effects on HD cells. Exemplary nucleic acids of the invention comprise SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:27, SEQ ID NO:29 or SEQ ID NO:31. Preferred nucleic acids

30   of the invention comprise SEQ ID NO:1, SEQ ID NO:9, SEQ ID NO:17, or SEQ ID NO:25. (See Table 1 at pages 9-10, *supra*, for identification of the domain of AC10 or HeFi-1 to which these sequence identifiers correspond).

The invention also encompasses nucleic acids that hybridize under stringent, moderate or low stringency hybridization conditions, to nucleic acids of the invention,

35   preferably, nucleic acids encoding an antibody of the invention.

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By way of example and not limitation, procedures using such conditions of low stringency for regions of hybridization of over 90 nucleotides are as follows (*see also* Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. U.S.A. 78,:6789-6792). Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC,

5 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 10^6 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at

10 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and re-exposed to film. Other conditions of low stringency which may be used are well known in the art (*e.g.*, as employed for cross-species

15 hybridizations).

Also, by way of example and not limitation, procedures using such conditions of high stringency for regions of hybridization of over 90 nucleotides are as follows. Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP,

20 0.02% Ficoll, 0.02% BSA, and 500 μg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h at 65°C in prehybridization mixture containing 100 μg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C for 45 min before autoradiography.

25 Other conditions of high stringency which may be used depend on the nature of the nucleic acid (*e.g.* length, GC content, *etc.*) and the purpose of the hybridization (detection, amplification, *etc.*) and are well known in the art. For example, stringent hybridization of a nucleic acid of approximately 15-40 bases to a complementary sequence in the polymerase chain reaction (PCR) is done under the following conditions: a salt

30 concentration of 50 mM KCl, a buffer concentration of 10 mM Tris-HCl, a Mg^{2+} concentration of 1.5 mM, a pH of 7-7.5 and an annealing temperature of 55-60°C.

In another specific embodiment, a nucleic acid which is hybridizable to a nucleic acid of the invention acid, or its complement, under conditions of moderate stringency is provided. Selection of appropriate conditions for such stringencies is well

35 known in the art (*see e.g.*, Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *see*

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also, Ausubel *et al.*, eds., in the Current Protocols in Molecular Biology series of laboratory technique manuals, © 1987-1997, Current Protocols, © 1994-1997 John Wiley and Sons, Inc.).

5   The nucleic acids of the invention may be obtained, and the nucleotide sequence of the nucleic acids determined, by any method known in the art. For example, if the nucleotide sequence of the protein is known, a nucleic acid encoding the antibody may be assembled from chemically synthesized oligonucleotides (*e.g.*, as described in Kutmeier *et al.*, 1994, BioTechniques 17:242), which, briefly, involves the synthesis of overlapping oligonucleotides containing portions of the sequence encoding the protein, annealing and

10   ligating of those oligonucleotides, and then amplification of the ligated oligonucleotides by PCR.

     Alternatively, a nucleic acid encoding a protein of the invention may be generated from nucleic acid from a suitable source. If a clone containing a nucleic acid encoding a particular protein is not available, but the sequence of the protein molecule is

15   known, a nucleic acid encoding the protein may be chemically synthesized or obtained from a suitable source (*e.g.*, a cDNA library such as an antibody cDNA library or a cDNA library generated from, or nucleic acid, preferably poly A+ RNA, isolated from, any tissue or cells expressing the protein. If the protein is an antibody, the library source can be hybridoma cells selected to express the antibody of the invention) by PCR amplification using

20   synthetic primers hybridizable to the 3' and 5' ends of the sequence or by cloning using an oligonucleotide probe specific for the particular gene sequence to identify, *e.g.*, a cDNA clone from a cDNA library that encodes the protein. Amplified nucleic acids generated by PCR may then be cloned into replicable cloning vectors using any method well known in the art.

25   Once the nucleotide sequence and corresponding amino acid sequence of the antibody is determined, the nucleotide sequence of the protein may be manipulated using methods well known in the art for the manipulation of nucleotide sequences, *e.g.*, recombinant DNA techniques, site directed mutagenesis, PCR, *etc.* (see, for example, the techniques described in Sambrook *et al.*, 1990, Molecular Cloning, A Laboratory Manual,

30   2d Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY and Ausubel *et al.*, eds., 1998, Current Protocols in Molecular Biology, John Wiley & Sons, NY, which are both incorporated by reference herein in their entireties *)*, to generate antibodies having a different amino acid sequence, for example to create amino acid substitutions, deletions, and/or insertions.

35   In a specific embodiment, the protein is an antibody, and the amino acid sequence of the heavy and/or light chain variable domains may be inspected to identify the

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sequences of the CDRs by methods that are well know in the art, *e.g.*, by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. Using routine recombinant DNA techniques, one or more of the CDRs may be inserted within framework regions, *e.g.*, into human

5 framework regions to humanize a non-human antibody, as described *supra*. The framework regions may be naturally occurring or consensus framework regions, and are preferably human framework regions (*see, e.g.*, Chothia *et al.*, 1998, J. Mol. Biol. 278:457-479 for a listing of human framework regions). The nucleic acid generated by the combination of the framework regions and CDRs encodes an antibody that specifically binds CD30 and exerts

10 a cytostatic and/or cytotoxic effect on HD cells. Preferably, as discussed *supra*, one or more amino acid substitutions may be made within the framework regions, and, preferably, the amino acid substitutions improve binding of the antibody to CD30 and/or to enhance the cytostatic and/or cytotoxic effect of the antibody. Additionally, such methods may be used to make amino acid substitutions or deletions of one or more variable region cysteine

15 residues participating in an intrachain disulfide bond to generate antibody molecules lacking one or more intrachain disulfide bonds. Other alterations to the nucleic acid are encompassed by the present invention and within the skill of the art.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison *et al.*, 1984, Proc. Natl. Acad. Sci. 81:851-855; Neuberger *et al.*,

20 1984, Nature 312:604-608 ; Takeda *et al.*, 1985, Nature 314:452-454) by splicing genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. As described *supra*, a chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb

25 and a human immunoglobulin constant region, *e.g.*, humanized antibodies.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778; Bird, 1988, Science 242:423- 42; Huston *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward *et al.*, 1989, Nature 334:544-54) can be adapted to produce single chain antibodies. Single chain antibodies are formed by

30 linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain protein. Techniques for the assembly of functional Fv fragments in *E. coli* may also be used (Skerra *et al.*, 1988, Science 242:1038- 1041).

35

5.5 SEQUENCES RELATED TO AC10 AND HeFi-1

The present invention further encompasses proteins and nucleic acids comprising a region of homology to CDRs of AC10 and HeFi-1, or the coding regions therefor, respectively. In various embodiments, the region of homology is characterized by at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity with the corresponding region of AC10 or HeFi-1.

In one embodiment, the present invention provides a protein with a region of homology to a CDR of HeFi-1 (SEQ ID NO:20, SEQ ID NO:22; SEQ ID NO:24; SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32). In another embodiment, the present invention provides a protein with a region of homology to a CDR of AC10 (SEQ ID NO:4; SEQ ID NO:6; SEQ ID NO:8; SEQ ID NO:12; SEQ ID NO:14; or SEQ ID NO:16).

In another embodiment, the present invention provides a nucleic acid with a region of homology to a CDR coding region of HeFi-1 (SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:27, SEQ ID NO:29 or SEQ ID NO:31). In yet another embodiment, the present invention provides a nucleic acid with a region of homology to a CDR coding region of AC10 (SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15).

The present invention further encompasses proteins and nucleic acids comprising a region of homology to the variable regions of AC10 and HeFi-1, or the coding region therefor, respectively. In various embodiments, the region of homology is characterized by at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity with the corresponding region of AC10 or HeFi-1.

In one embodiment, the present invention provides a protein with a region of homology to a variable region of HeFi-1 (SEQ ID NO:18 or SEQ ID NO: 26). In another embodiment, the present invention provides a protein with a region of homology to a variable region of AC10 (SEQ ID NO: 2 or SEQ ID NO: 10).

In one embodiment, the present invention provides a nucleic acid with a region of homology to a variable region coding region of HeFi-1 (SEQ ID NO:17 or SEQ ID NO:25). In another embodiment, the present invention provides a nucleic with a region of homology to a variable region coding region of AC10 (SEQ ID NO:1 or SEQ ID NO:9).

To determine the percent identity of two amino acid sequences or of two nucleic acids, e.g. between the sequences of an AC10 or HeFi-1 variable region and sequences from other proteins with regions of homology to the AC10 or HeFi-1 variable

region, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then

5 compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions (*e.g.*, overlapping positions) x 100). In one

10 embodiment, the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin

15 and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.*, 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid encoding a SCA-1 modifier protein. BLAST protein searches can be

20 performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a SCA-1 modifier protein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing

25 BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG

30 sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti, 1994, Comput. Appl. Biosci., 10:3-5; and FASTA described in Pearson and Lipman, 1988, Proc.

35 Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a control option that sets the sensitivity and speed of the search. If ktup=2, similar regions in the two sequences being compared are

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found by looking at pairs of aligned residues; if ktup=1, single aligned amino acids are examined. ktup can be set to 2 or 1 for protein sequences, or from 1 to 6 for DNA sequences. The default if ktup is not specified is 2 for proteins and 6 for DNA. For a further description of FASTA parameters, see

5   http://bioweb.pasteur.fr/docs/man/man/fasta.1.html#sect2, the contents of which are incorporated herein by reference.

Alternatively, protein sequence alignment may be carried out using the CLUSTAL W algorithm, as described by Higgins *et al.*, 1996, Methods Enzymol. 266:383-402.

10   The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

5.6   METHODS OF PRODUCING THE PROTEINS OF THE INVENTION

15   The proteins, including antibodies, of the invention can be produced by any method known in the art for the synthesis of proteins, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Recombinant expression of a protein of the invention, including a fragment, derivative or analog thereof, (*e.g.*, a heavy or light chain of an antibody of the invention)
20   requires construction of an expression vector containing a nucleic acid that encodes the protein. Once a nucleic acid encoding a protein of the invention has been obtained, the vector for the production of the protein molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a nucleic acid containing nucleotide sequence encoding said protein are
25   described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding a protein of the
30   invention operably linked to a promoter. Wherein the protein is an antibody, the nucleotide sequence may encode a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (*see, e.g.,* PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable
35   domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

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The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a protein of the invention. Thus, the invention encompasses host cells containing a nucleic acid encoding a protein of the invention, operably linked to a heterologous promoter. In

5   preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the proteins molecules of the invention. Such host-expression systems represent vehicles by

10   which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express a protein of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g., E. coli, B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors

15   containing antibody coding sequences; yeast (*e.g.*, Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or

20   transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses (*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial

25   cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecules, are used for the expression of a recombinant protein of the invention. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system

30   for proteins of the invention (Foecking *et al.*, 1986, Gene 45:101; Cockett *et al.*, 1990, Bio/Technology 8:2).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the folding and post-translation modification requirements protein being expressed. Where possible, when a large quantity of such a

35   protein is to be produced, for the generation of pharmaceutical compositions comprising a protein of the invention, vectors which direct the expression of high levels of fusion protein

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products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO 1. $\underline{2}$:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye &

5   Inouye, 1985, Nucleic Acids Res. $\underline{13}$:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. $\underline{24}$:5503-5509); and the like. pGEX vectors may also be used to express fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors

10   are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-

15   essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the coding sequence of the protein of the invention may be ligated to an adenovirus transcription/translation

20   control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non- essential region of the viral genome (*e.g.*, region El or E3) will result in a recombinant virus that is viable and capable of expressing the protein of the invention in infected hosts. (*See, e.g.*, Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 8 $\underline{1}$:355-359).

25   Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and

30   synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner *et al.*, 1987, Methods in Enzymol. $\underline{153}$:51-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion

35   desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein of the invention. Different host

cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper

5 processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, Hela, COS, MDCK, 293, 3T3, and W138.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the protein of the

10 invention may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media,

15 and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the protein of the invention.

20 A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1992, Proc. Natl. Acad. Sci. USA 48:202), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:8-17) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite

25 resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, Proc. Natl. Acad. Sci. USA 77:357; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Clinical Pharmacy 12:488-505; Wu

30 and Wu, 1991, Biotherapy 3:87-95 ; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932 ; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62: 191-217; May, 1993, TIB TECH 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the

35 desired recombinant clone, and such methods are described, for example, in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler,

Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli *et al.* (eds), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1, which are incorporated by reference herein in their entireties.

5   The expression levels of a protein of the invention can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use of Vectors Based on Gene Amplification for the Expression of Cloned Genes in Mammalian Cells in DNY Cloning", Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of

10   host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the protein of the invention will also increase (Crouse *et al.*, 1983, Mol. Cell. Biol. 3:257).

        Wherein the protein of the invention is an antibody, the host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy

15   chain derived protein and the second vector encoding a light chain derived protein. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain proteins. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain proteins. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain

20   (Proudfoot, 1986, Nature 322:52 (1986); Kohler, 1980, Proc. Natl. Acad. Sci. USA 77:2 197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

        Once a protein molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method

25   known in the art for purification of proteins, for example, by chromatography (*e.g.*, ion exchange; affinity, particularly by affinity for the specific antigen, Protein A (for antibody molecules, or affinity for a heterologous fusion partner wherein the protein is a fusion protein; and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

30   The present invention encompasses CD3-binding proteins recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugation) to heterologous proteins (of preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or at least 100 amino acids) of the present invention to generate fusion proteins. The fusion does not necessarily need to be direct, but may occur through linker sequences.

35   The present invention further includes compositions comprising proteins of the invention fused or conjugated to antibody domains other than the variable regions. For

example, the proteins of the invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a protein of the invention may comprise the constant region, hinge region, CH 1 domain, CR2 domain, and CH3 domain or any combination of whole domains or portions thereof. The proteins may also be fused or

5   conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the proteins of the invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the proteins to portions of IgA and IgM. Methods for fusing or conjugating the proteins of the invention to antibody portions are known in the art. *See, e.g.,* U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046;

10   5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 9 1/06570; Ashkenazi *et al.*, 1991, Proc. Nat. Acad. Sci. USA 88:10535-10539; Zheng *et al.*, 1995, J. Immunol. 154:5590-5600; and Vil *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:11337- 11341 (said references incorporated by reference in their entireties).

15

5.7   CONJUGATES AND FUSION PROTEINS

As discussed, *supra*, the proteins of the invention encompass proteins that bind to CD30 and exert a cytostatic and/or cytotoxic effect on HD cells, and that are further fused or conjugated to heterologous proteins or cytotoxic agents.

20   The present invention thus provides for treatment of Hodgkin's Disease by administration of a protein or nucleic acid of the invention. Proteins of the invention include but are not limited to: AC10 and HeFi-1 proteins, antibodies and analogs and derivatives thereof (*e.g.*, as described herein above); the nucleic acids of the invention include but are not limited to nucleic acids encoding such AC10 and HeFi-1 proteins,

25   antibodies and analogs or derivatives (*e.g.*, as described herein above).

In certain embodiments of the invention, a protein or nucleic acid of the invention may be chemically modified to improve its cytotoxic and/or cytostatic properties. For example, a protein of the invention can be administered as a conjugate. Particularly suitable moieties for conjugation to proteins of the invention are chemotherapeutic agents,

30   pro-drug converting enzymes, radioactive isotopes or compounds, or toxins. Alternatively, a nucleic acid of the invention may be modified to functionally couple the coding sequence of a pro-drug converting enzyme with the coding sequence of a protein of the invention, such that a fusion protein comprising the functionally active pro-drug converting enzyme and protein of the invention is expressed in the subject upon administration of the nucleic

35   acid in accordance with the gene therapy methods described in Section 5.7, *infra*.

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In one embodiment, a protein of the invention is fused to a marker sequence, such as a peptide, to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which

5 are commercially available. As described in Gentz *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, 1984, Cell 37:767) and the "flag" tag. Such fusion proteins can be generated

10 by standard recombinant methods known to those of skill in the art.

In another embodiment, the proteins of the invention are fused or conjugated to a therapeutic agent. For example, a protein of the invention may be conjugated to a cytotoxic agent such as a chemotherapeutic agent, a toxin (*e.g.*, a cytostatic or cytocidal agent), or a radionuclide (*e.g.*, alpha-emitters such as, for example, ^{212}Bi, ^{211}At, or beta-

15 emitters such as, for example, ^{131}I, ^{90}Y, or ^{67}Cu).

Drugs such as methotrexate (Endo *et al.*, 1987, Cancer Research 47:1076-1080), daunomycin (Gallego *et al.*, 1984, Int. J. Cancer. 33:737-744), mitomycin C (MMC) (Ohkawa *et al.*, 1986, Cancer Immunol. Immunother. 23:81-86) and vinca alkaloids (Rowland *et al.*, 1986, Cancer Immunol Immunother. 21:183-187) have been attached to

20 antibodies and the derived conjugates have been investigated for anti-tumor activities. Care should be taken in the generation of chemotherapeutic agent conjugates to ensure that the activity of the drug and/or protein does not diminish as a result of the conjugation process.

Examples of chemotherapeutic agents include the following non-mutually exclusive classes of chemotherapeutic agents: alkylating agents, anthracyclines, antibiotics,

25 antifolates, antimetabolites, antitubulin agents, auristatins, chemotherapy sensitizers, DNA minor groove binders, DNA replication inhibitors, duocarmycins, etoposides, fluorinated pyrimidines, lexitropsins, nitrosoureas, platinols, purine antimetabolites, puromycins, radiation sensitizers, steroids, taxanes, topoisomerase inhibitors, and vinca alkaloids. Examples of individual chemotherapeutics that can be conjugated to a nucleic acid or

30 protein of the invention include but are not limited to an androgen, anthramycin (AMC), asparaginase, 5-azacytidine, azathioprine, bleomycin, busulfan, buthionine sulfoximine, camptothecin, carboplatin, carmustine (BSNU), CC-1065, chlorambucil, cisplatin, colchicine, cyclophosphamide, cytarabine, cytidine arabinoside, cytochalasin B, dacarbazine, dactinomycin (formerly actinomycin), daunorubicin, decarbazine, docetaxel,

35 doxorubicin, an estrogen, 5-fluordeoxyuridine, 5-fluorouracil, gramicidin D, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine (CCNU), mechlorethamine, melphalan, 6-

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mercaptopurine, methotrexate, mithramycin, mitomycin C, mitoxantrone, nitroimidazole, paclitaxel, plicamycin, procarbizine, streptozotocin, tenoposide, 6-thioguanine, thioTEPA, topotecan, vinblastine, vincristine, vinorelbine, VP-16 and VM-26.

The conjugates of the invention used for enhancing the therapeutic effect of
5   the protein of the invention include non-classical therapeutic agents such as toxins. Such toxins include, for example, abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin.

Techniques for conjugating such therapeutic moieties to proteins, and in particular to antibodies, are well known, see, e.g., Arnon et al., "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer
10 Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R. Liss, Inc., 1985); Hellstrom et al., "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd ed.), Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc., 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera et al. (eds.), pp. 475-506 (1985); "Analysis, Results, And
15 Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin et al. (eds.), pp. 303-16 (Academic Press 1985), and Thorpe et al., 1982, Immunol. Rev. 62:119-58.

Alternatively, an antibody of the invention can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No.
20 4,676,980, which is incorporated herein by reference in its entirety.

As discussed above, in certain embodiments of the invention, a protein of the invention can be co-administered with a pro-drug converting enzyme. The pro-drug converting enzyme can be expressed as a fusion protein with or conjugated to a protein of the invention. Exemplary pro-drug converting enzymes are carboxypeptidase G2, beta-
25 glucuronidase, penicillin-V-amidase, penicillin-G-amidase, β-lactamase, β-glucosidase, nitroreductase and carboxypeptidase A.

5.8   GENE THERAPY
In a specific embodiment, nucleic acids of the invention are administered to
30 treat, inhibit or prevent HD. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.
35   For general reviews of the methods of gene therapy, see, Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev,

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1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 1, 1(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the therapeutic comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue- specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438. In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid- carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, for example by constructing them as part of an appropriate nucleic acid expression vector and administering the vector so that the nucleic acid sequences become intracellular. Gene therapy vectors can be administered by infection using defective or attenuated retrovirals or other viral vectors (*see, e.g.*, U.S. Patent No. 4,980,286); direct injection of naked DNA; use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); coating with lipids or cell-surface receptors or transfecting agents; encapsulation in liposomes, microparticles, or microcapsules; administration in linkage to a peptide which is known to enter the nucleus; administration in linkage to a ligand subject to receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types

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specifically expressing the receptors); *etc.* In another embodiment, nucleic acid-ligand complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression,

5   by targeting a specific receptor (*see, e.g.*, PCT Publications WO 92/06 180; WO 92/22635; W092/20316; W093/14188, and WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

10   In a specific embodiment, viral vectors that contain nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller *et al.*, 1993, Meth. Enzymol. 217:581-599). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be

15   used in gene therapy are cloned into one or more vectors, thereby facilitating delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen *et al.*, 1994, Biotherapy 6:29 1-302, which describes the use of a retroviral vector to deliver the mdr 1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy

20   are: Clowes *et al.*, 1994, J. Clin. Invest. 93:644-651; Klein *et al.*, 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

    Another approach to gene therapy involves transferring a gene, *e.g.* an AC10 or HeFi-1 gene, to cells in tissue culture by such methods as electroporation,

25   lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

    In this embodiment, the nucleic acid is introduced into a cell prior to

30   administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell mediated gene transfer, spheroplast fusion, *etc.* Numerous techniques are known in the art

35   for the introduction of foreign genes into cells (*see, e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen *et al.*, 1993, Meth. Enzymol. 217:618-644; Cline, 1985,

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Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and

5   expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, *etc.*, and can be determined by one skilled in the

10   art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to fibroblasts; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in

15   particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, *etc.*

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy,

20   nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see *e.g.*

25   PCT Publication WO 94/08598; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, 1980, Meth. Cell Bio. 21A:229; and Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of

30   the appropriate inducer of transcription.

The compounds or pharmaceutical compositions of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays to demonstrate the therapeutic or prophylactic utility of an protein or pharmaceutical composition include determining the

35   effect of the protein or pharmaceutical composition on a Hodgkin's cell line or a tissue sample from a patient with Hodgkin's Disease. The cytotoxic and/or cytostatic effect of the

protein or composition on the Hodgkin's cell line and/or tissue sample can be determined utilizing techniques known to those of skill in the art. A preferred method, described in Section 6 *infra*, entails contacting a culture of the Hodgkin's Disease cell line grown at a density of approximately of about 5,000 cells in a 0.33 cm^2 of culture area for a period of 72

5 hours with the protein or pharmaceutical composition, exposing the culture to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period, and measuring the incorporation of ^{3}H-thymidine into cells of the culture. The protein or pharmaceutical composition has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line and is useful for the treatment or prevention of Hodgkin's Disease if the cells of the culture have

10 reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein or pharmaceutical composition. Alternatively, *in vitro* assays which can be used to determine whether administration of a specific protein or pharmaceutical composition is indicated, include *in vitro* cell culture assays in which a tissue sample from a Hodgkin's Disease patient is grown

15 in culture, and exposed to or otherwise a protein or pharmaceutical composition, and the effect of such compound upon the Hodgkin's tissue sample is observed.

5.9 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

20 The invention provides methods of treatment and prophylaxis by administration to a subject of an effective amount of a CD30-binding protein which has a cytotoxic or cytostatic effect on Hodgkin's Disease cells (*i.e.*, a protein of the invention), a nucleic acid encoding said CD30-binding protein (*i.e.*, a nucleic acid of the invention), or a pharmaceutical composition comprising a protein or nucleic acid of the invention

25 (hereinafter, a pharmaceutical of the invention). According to the present invention, treatment of HD encompasses the treatment of patients already diagnosed as HD at any clinical stage; such treatment resulting in delaying tumor growth; and/or promoting tumor regression.

In a preferred embodiment, the protein of the invention is the monoclonal

30 antibody AC10 or HeFi-1 or a fragment or derivative thereof. In a preferred aspect, a pharmaceutical of the invention comprises a substantially purified protein or nucleic acid of the invention (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, *etc.*, and is preferably a mammal,

35 and most preferably human.

Formulations and methods of administration that can be employed are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a nucleic
5   acid or protein of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, *etc.* Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous,
10   intranasal, epidural, and oral routes. Nucleic acids and proteins of the invention may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents such as chemotherapeutic agents (see Section). Administration can be systemic or
15   local.

In a specific embodiment, it may be desirable to administer the nucleic acid or protein of the invention by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including a membrane, such as a sialastic membrane, or a fiber. Preferably, when
20   administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990, Science 249:1527-1533; Treat *et al.*, 1989, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and
25   Fidler (eds.), Liss, New York, pp. 353- 365; Lopez-Berestein, ibid., pp. 317-327; see generally, *ibid.*)

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1989, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery
30   88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy *et al.*, 1985,
35   Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105).

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Other controlled release systems are discussed in the review by Langer, 1990, Science 249:1527-1533.

In a specific embodiment where a nucleic acid of the invention is administered, the nucleic acid can be administered *in vivo* to promote expression of its

5   encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (*see* U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox- like peptide which is

10 known to enter the nucleus (*see e.g.*, Joliot *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), *etc.* Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

As alluded to above, the present invention also provides pharmaceutical compositions (pharmaceuticals of the invention). Such compositions comprise a

15 therapeutically effective amount of a nucleic acid or protein of the invention, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant,

20 excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be

25 employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

30 These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate,

35 *etc.* Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a

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therapeutically effective amount of the nucleic acid or protein of the invention, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the pharmaceutical of the invention is formulated
5   in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical of the invention may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are
10 supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the pharmaceutical of the invention is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the pharmaceutical of the
15 invention is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The amount of the nucleic acid or protein of the invention which will be effective in the treatment or prevention of HD can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal
20 dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the stage of HD, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

25   5.10   **KITS**
The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with a nucleic acid or protein of the invention and optionally one or more pharmaceutical carriers. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of
30 pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

In one embodiment, a kit comprises a purified protein of the invention. In a preferred mode of the embodiment, the protein is an antibody. The protein may be conjugated to a radionuclide or chemotherapeutic agent. The kit optionally further
35 comprises a pharmaceutical carrier.

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In another embodiment, a kit of the invention comprises a nucleic acid of the invention, or a host cell comprising a nucleic acid of the invention, operably linked to a promoter for recombinant expression.

5

5.11 EFFECTIVE DOSE

Toxicity and therapeutic efficacy of the proteins of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and

10 therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Proteins that exhibit large therapeutic indices are preferred. While proteins that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such proteins to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

15 The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such proteins lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the

20 invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in

25 plasma may be measured, for example, by high performance liquid chromatography.

Generally, the dosage of a protein of the invention in a pharmaceutical of the invention administered to a Hodgkin's Disease patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of

30 the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign proteins. Thus, lower dosages of humanized, chimeric or human antibodies and less frequent administration is often possible.

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5.12 FORMULATIONS

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

Thus, the proteins and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate) lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the proteins for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

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The proteins may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multidose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions

5 in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The proteins may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as

10 cocoa butter or other glycerides.

In addition to the formulations described previously, the proteins may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the proteins may be formulated with suitable polymeric or

15 hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or

20 dispenser device may be accompanied by instructions for administration preferably for administration to a human.

5.13   COMBINATION THERAPY FOR TREATMENT OF HODGKIN'S DISEASE

25
The nucleic acids and proteins of the invention can be administered together with treatment with irradiation or one or more chemotherapeutic agents.

For irradiation treatment, the irradiation can be gamma rays or X-rays. For a general overview of radiation therapy, see Hellman, Chapter 12: Principles of Radiation Therapy Cancer, in: Principles and Practice of Oncology, DeVita *et al.*, eds., 2nd. Ed., J.B.

30 Lippencott Company, Philadelphia.

Useful classes of chemotherapeutic agents include, but are not limited to, the following non-mutually exclusive classes of agents: alkylating agents, anthracyclines, antibiotics, antifolates, antimetabolites, antitubulin agents, auristatins, chemotherapy sensitizers, DNA minor groove binders, DNA replication inhibitors, duocarmycins,

35 etoposides, fluorinated pyrimidines, lexitropsins, nitrosoureas, platinols, purine antimetabolites, puromycins, radiation sensitizers, steroids, taxanes, topoisomerase inhibitors, and vinca alkaloids. Individual chemotherapeutics encompassed by the

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invention include but are not limited to an androgen, anthramycin (AMC), asparaginase, 5-azacytidine, azathioprine, bleomycin, busulfan, buthionine sulfoximine, camptothecin, carboplatin, carmustine (BSNU), CC-1065, chlorambucil, cisplatin, colchicine, cyclophosphamide, cytarabine, cytidine arabinoside, cytochalasin B, dacarbazine,

5   dactinomycin (formerly actinomycin), daunorubicin, decarbazine, docetaxel, doxorubicin, an estrogen, 5-fluordeoxyuridine, 5-fluorouracil, gramicidin D, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine (CCNU), mechlorethamine, melphalan, 6-mercaptopurine, methotrexate, mithramycin, mitomycin C, mitoxantrone, nitroimidazole, paclitaxel, plicamycin, procarbizine, streptozotocin, tenoposide, 6-thioguanine, thioTEPA, topotecan,

10   vinblastine, vincristine, vinorelbine, VP-16 and VM-26.

In a specific embodiment, a nucleic acid or protein of the invention is administered concurrently with radiation therapy or one or more chemotherapeutic agents. In another specific embodiment, chemotherapy or radiation therapy is administered prior or subsequent to administration of a nucleic acid or protein of the invention, by at least an hour

15   and up to several months, for example at least an hour, five hours, 12 hours, a day, a week, a month, or three months, prior or subsequent to administration of a nucleic acid or protein of the invention.

In a specific embodiment in which a protein of the invention is conjugated to a pro-drug converting enzyme, or in which a nucleic acid of the invention encodes a fusion

20   protein comprising a pro-drug converting enzyme, the protein or nucleic acid is administered with a pro-drug. Administration of the pro-drug can be concurrent with administration of the nucleic acid or protein of the invention, or, more preferably, follows the administration of the nucleic acid or protein of the invention by at least an hour to up to one week, for example about five hours, 12 hours, or a day. Depending on the pro-drug

25   converting enzyme administered, the pro-drug can be a benzoic acid mustard, an aniline mustard, a phenol mustard, p-hydroxyaniline mustard-glucuronide, epirubicin-glucuronide, adriamycin-N phenoxyaceryl, N-(4'-hydroxyphenyl acetyl)-palytoxin doxorubicin, melphalan, nitrogen mustard-cephalosporin, β-phenylenediamine, vinblastine derivative-cephalosporin, cephalosporin mustard, cyanophenylmethyl-β-D-gluco-pyranosiduronic

30   acid, 5-(adaridin-1-yl-)2, 4-dinitrobenzamide, or methotrexate-alanine.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the

35   scope of the appended claims.

The invention is further described in the following examples which are in no

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way intended to limit the scope of the invention.

6. EXAMPLE: ANTI-CD30 MONOCLONAL ANTIBODIES AC10 AND HeFi-1 INHIBIT THE GROWTH OF CD30-EXPRESSING HODGKIN'S DISEASE CELL LINES

6.1 MATERIALS AND METHODS

Cells and culture conditions: The CD30 expressing cell lines, L540, HDLM2, L428, KM-H2 and Karpas 299. were obtained from the German Collection of Microorganisms and Cell Cultures/DSMZ in Braunschweig, Germany. The Hodgkin's cell line L540cy was a provided by Dr. V. Diehl of the University of Cologne, Cologne, Germany. The cell lines were maintained in the recommended media formulations and subcultured every 3-4 days.

Reagents and antibodies: Anti-CD30 monoclonal antibody hybridoma line AC10 was described by Bowen *et al.* (Bowen *et al.*, 1993, J. Immunol. 151:5896-5906). Purified antibody was isolated from serum-free supernatants using a protein-G immunoaffinity column. The resulting AC10 antibody was determined to be > 97% monomeric by size exclusion chromatography. The monoclonal antibody HeFi-1 has been previously described and was provided by Dr. T. Hecht, NCI, Bethesda, MD. HeFi-1 mAb was demonstrated by size exclusion chromatography to be greater than 98% monomer.

Proliferation assays: CD30 expressing cell lines were cultured in flat-bottom 96-well plates at a density of 50,000 or 5,000 cells/well in growth media (RPMI with10% fetal bovine serum (FBS) for cell lines L428, KM-H2 and Karpas 299, and RPMI/20%FBS for cell lines HDLM-2 and L540. The cell lines were cultured in the absence or presence of cross-linked soluble anti-CD30 mAbs or immobilized anti-CD30 mAbs, as described below.

Antibody cross-linking in solution: To cross-link the anti-CD30 antibodies in solution, various dilutions of AC10 or HeFi- 1 were titrated into 96-well flat bottom tissue culture plates in the absence or presence of 20 μg/ml polyclonal goat anti-mouse IgG antibodies. Hodgkin's disease cell lines were then added to the plates at either 50,000 or 5,000 cells/well. The plates were incubated at 37°C for 72 hours and were labeled with ^{3}H-thymidine, 1 μCi/well, for the final 5 hours.

Antibody immobilization: Antibody immobilization was obtained by coating wells with antibody in 50 mmol/L Tris buffer (pH 8.5) for 18 hours at 4°C. Prior to the addition of cells, wells were washed twice with PBS to remove unbound mAb. 50,000 or 5,000 cells in a total volume of 200 μl were added to each well. Proliferation was determined by uptake of ^{3}H-thymidine (0.5 μCi/well) during the final 8 hours of a 72 hour culture period.

6.2 RESULTS

To evaluate the biologic activity of anti-CD30 mAbs, CD30-expressing HD cell lines (50,000 cells/well) were cultured in the presence of immobilized anti-CD30 mAb
5 AC10. mAb AC10 demonstrated inhibition of cell growth of T-cell-like (L540 and HDLM-2) or B-cell-like (L428 and KM-H2) HD lines (FIG. 1). Ki-1, which was previously shown to have no effect on HD cell lines (Gruss *et al.*, 1996, Blood 83:2045-2056), was used as a control.

To further evaluate the activity of AC10, a second series of assays were
10 performed. In order to assess the activity of the AC10 during a period of logarithmic tumor cell growth, the cell density of the cultures was decreased to provide more optimal growth conditions. To that end, HD cell lines were cultured in flat-bottom 96 well plates at a density of 5,000 cells/well in the presence or absence of mAb AC10. AC10 demonstrated growth inhibition of all four HD cell lines tested (L540, HDLM-2, L428 and KM-H2; FIG.
15 2).

In another set of experiments, HD cell lines were incubated with soluble AC10 or HeFi-1 that were cross-linked in solution by the addition of soluble goat anti-mouse IgG antibodies. Under these cross-liking conditions, all four HD cell lines, when plated at 5×10^4 cell/well, were growth inhibited by AC10 and HeFi-1 (FIG. 3). When the
20 cells were plated at 5×10^3 cell/well, all four HD cell lines were growth inhibited by AC10, while three of the four cell lines, HDLM-2, L540, and L428, were growth inhibited by HeFi-1 (FIG. 4).

The data resulting from the experiments testing the effects of AC10 and HeFi-1 on CD30-expressing tumor cell lines are summarized in Table 2, *infra*. Table 2
25 further provides a comparison of the anti-tumor activity of AC10 and HeFi-1 with that of mAb M44.

		Inhibition of Growth by		
Cell Line	Cell Type	M44[a]	HeFi-1	AC10
Karpas 299	ALCL	+	+	+
Michel	ALCL	+	ND	ND
KM-H2	HD (B cell phenotype)	-	+	+
L428	HD (B cell phenotype)	-	+	+
HDLM-2	HD (T cell phenotype)	-	+	+

| L540 | HD (T cell phenotype) | - | + | + |

Table 2. Cytostatic and/or cytotoxic activity of signaling anti-CD30 mAbs on CD30-expressing malignant cell lines

Taken together, these data indicate that mAbs AC10 and HeFi-1 are distinguished from the previously described anti-CD30 mAbs by their ability to inhibit the growth of CD30-expressing HD lines. It is of interest to note that Hubinger *et al.* recently evaluated the activity of the anti-CD30 mAb M44, in immobilized form, in a proliferation assay utilizing 5,000 cells/well. Under these conditions, M44 inhibited the growth of the CD30-expressing ALCL line, Karpas 299 but not the HD cell line HDLM-2 (Hubinger *et al.*, 1999, Exp. Hematol. 27(12):1796-805).

7. AC10 ENHANCES THE CYTOTOXIC EFFECT OF CHEMOTHERAPEUTICS ON HODGKIN'S DISEASE CELL LINES

7.1 MATERIALS AND METHODS

L428 cells were cultured for 24 hours in the presence or absence of 0.1 µg/ml anti-CD30 antibody, AC10, crosslinked by the addition of 20 µg/ml goat anti-mouse IgG antibodies. After the 24-hour culture period, the cells were harvested and washed with phosphate buffered saline (PBS). The cells were then plated into 96-well flat-bottom tissue culture plates at 5×10^3 cells/well and mixed with various dilutions of chemotherapeutic drugs. After a 1-hour exposure to the drugs the cells were washed twice, followed by the addition of fresh culture media. The plates were then incubated at 37°C for 72 hours followed by a 4-hour incubation with 0.5 µCi/well ^{3}H-thymidine. The inhibition of growth was determined by comparing the amount of ^{3}H-thymidine incorporated into treated cells to the amount incorporated into untreated control cells.

7.2 RESULTS

To evaluate the effect of the anti-CD30 mAb in combination with chemotherapeutic drugs, L428 cells were incubated for 24 hours in either the absence of antibody or the presence of AC10 at 0.1 µg/ml with 20 µg/ml goat anti-mouse IgG to provide crosslinking for the primary antibody. After this incubation the cells were plated into 96-well tissue culture plates at 5×10^3 cells/well in the presence of dilutions of chemotherapeutic drugs including doxorubicin, cisplatin, and etoposide (Table 3). The EC_{50}, concentration of drug needed to inhibit the incorporation of ^{3}H-thymidine by 50%

compared to untreated control cells, was then determined for cells treated with the drugs alone or the combinations of drug and antibody. For doxorubicin, incubation with AC10 decreased the EC_{50} on L428 cells (*i.e.* decreased the amount of drug necessary to inhibit 50% of DNA synthesis) from approximately 45 nM (doxorubicin alone) to approximately

5 9nM, for cisplatin AC10 decreased the EC_{50} from ~1,500 nM to ~500 nM, and for etoposide AC10 decreased the EC_{50} from ~1,500 nM to ~600 nM.

	EC_{50}, nM	
Drug	with AC10	without AC10
Doxorubicin	45	9
Cisplatin	1,500	500
Etoposide	1,500	600

15 **Table 3:** AC10 enhances the effectiveness of chemotherapeutic drugs on the HD cell line L428.

8. ANTITUMOR ACTIVITY OF AC10 AND HeFi-1 IN DISSEMINATED AND LOCALIZED (SUBCUTANEOUS) L540CY HODGKIN'S DISEASE XENOGRAFTS

8.1 MATERIALS AND METHODS

20 *Human tumor xenograft models*: Female C.B-17 SCID mice, obtained from Taconic (Germantown, NY) at 4-6 weeks of age, were used for all efficacy studies. To establish xenograft models of Hodgkin's disease, L540cy (HD) cells were harvested from cell culture, washed in ice cold phosphate buffered saline (PBS), resuspended in PBS, and

25 maintained on ice until implantation. For disseminated disease models, mice were injected intravenously through the tail vein with 10^7 L540cy cells. Solid tumor xenografts were established by injecting mice subcutaneously (s.c.) with 2 x 10^7 L540cy cells. For therapeutic evaluation the indicated treatment doses and schedules were used.

Administration of AC10 and HeFi-1: Disseminated L540cy tumor bearing

30 mice received 10^7 cells through the tail vein on d0 followed by therapy initiated on d1. Treated mice received i.p. injections of either AC10 or HeFi-1 every two days for a total of 10 injections, q2dx10, at 1 mg/kg/injection.

For the subcutaneous L540cy model, mice were injected s.c. with 2 x 10^7 cells and were observed daily for solid tumor formation. When tumors were palpable, the

35 animals were randomly distributed into groups and received either AC10 or HeFi-1 q2dx10 at 2 mg/kg/injection.

8.2 RESULTS

AC10 and HeFi-1 were tested in L540cy Hodgkin's disease xenografted SCID mice, as described above. In the mouse population with disseminated L540cy tumors, all of the untreated control animals developed signs of severe disseminated disease such as hind limb paralysis or the formation of a solid tumor mass and had to be sacrificed (mean survival time = 37 days). In contrast, all of the mice that received either AC10 or HeFi-1 survived for > 46 days with no signs of disease (FIG. 5A).

With respect to the mouse population with subcutaneous L540cy tumors, while the untreated control tumors rapidly grew to > 450 mm^3, both mAbs significantly delayed tumor growth as shown in FIG. 5B.

The inventors have identified murine monoclonal antibodies (mAbs) which target the human CD30 receptor and display a profile of activity not previously described for other anti-CD30 mAbs. In unmodified form, these antibodies, AC10 and HeFi-1 inhibit the growth of HD and the ALCL line Karpas 299 and display *in vivo* antitumor activity in a tumor xenograft model of Hodgkin's disease.

9. ANTITUMOR ACTIVITY OF CHIMERIC AC10 IN SUBCUTANEOUS L540CY HODGKIN'S DISEASE XENOGRAFTS
9.1 MATERIALS AND METHODS

Chimeric AC10 (cAC 10) was generated via homologous recombination essentially as previously described using human IgG1-kappa heavy and light chain conversion vectors (Yarnold and Fell, 1994, Cancer Res. <u>54</u>: 506-512). These vectors were designed such that the murine immunoglobulin heavy and light chain constant region loci are excised and replaced by the human gamma 1 and kappa constant region loci via homologous recombination. The resulting chimeric hybridoma cell line expresses a chimeric antibody consisting of the heavy and light chain variable regions of the original monoclonal antibody and the human gamma 1 and kappa constant regions.

9.2 RESULTS

To evaluate the efficacy of cAC10 *in vivo*, SCID mice were implanted subcutaneously with L540cy cells as described above. When the tumors reached an average size of greater than 150 mm^3 the mice were divided into groups that were either untreated or treated with 2 mg/kg cAC 10 twice per week for a total of five injections. The tumors in the untreated mice rapidly grew to an average size of greater than 600 mm^3 (FIG. 6). In contrast, the average tumor size in the animals treated with cAC10 remained about the same size.

10. SPECIFIC EMBODIMENTS, CITATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of which are incorporated herein by reference in their entireties.

WHAT IS CLAIMED IS:

1.   A method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject, in an amount effective for said treatment or prevention, (a) an antibody that (i) immunospecifically binds CD30 and (ii) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line; and (b) a pharmaceutically acceptable carrier.

2.   The method of claim 1, wherein the antibody is human, humanized or chimeric.

3.   The method of claim 1, further comprising administering chemotherapy to said subject.

4.   The method of claim 1, wherein the antibody is conjugated to a cytotoxic agent.

5.   The method of claim 1, wherein the antibody is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody.

6.   The method of claim 4 or 5, further comprising administering chemotherapy to said subject.

7.   The method of claim 1, wherein the cytostatic or cytotoxic effect is determined by:
    (a)   contacting a culture of the Hodgkin's Disease cell line with the antibody, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;
    (b)   exposing the culture to 0.5 µCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and
    (c)   measuring the incorporation of ^{3}H-thymidine into cells of the culture, wherein the antibody has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the antibody.

8.   A method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and (b) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, which amount is effective for the
5   treatment or prevention of Hodgkin's Disease.

9.   A method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) comprises SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:14 or SEQ ID
10   NO:16, and (b) immunospecifically binds CD30, which amount is effective for the treatment or prevention of Hodgkin's Disease.

10.   A method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) comprises
15   SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32, and (b) immunospecifically binds CD30, which amount is effective for the treatment or prevention of Hodgkin's Disease.

11.   A method for the treatment or prevention of Hodgkin's Disease in a subject
20   comprising administering to the subject an amount of a protein, which protein (a) comprises an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID NO:10, and (b) immunospecifically binds CD30, which amount is effective for the treatment or prevention of Hodgkin's Disease.

25   12.   A method for the treatment or prevention of Hodgkin's Disease in a subject comprising administering to the subject an amount of a protein, which protein (a) comprises an amino acid sequence that has at least 95% identity to SEQ ID NO:18 or SEQ ID NO:26, and (b) immunospecifically binds CD30, which amount is effective for the treatment or prevention of Hodgkin's Disease.
30
13.   The method of any one of claims 8-12, wherein the protein is a human, humanized or chimeric antibody.

14.   The method of any one of claims 8-12, further comprising administering
35   chemotherapy to said subject.

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15.   The method of any one of claims 8-12, wherein the protein is conjugated to a cytotoxic agent.

16.   The method of any one of claims 8-12, wherein the protein is a fusion protein comprising the amino acid sequence of a second protein.

17.   The method of claim 15, further comprising administering chemotherapy to the subject.

18.   The method of claim 16, further comprising administering chemotherapy to the subject.

19.   The method of any one of claims 8-12, wherein the cytostatic or cytotoxic effect is determined by:
        (a)   contacting a culture of the Hodgkin's Disease cell line with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;
        (b)   exposing the culture to 0.5 µCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and
        (c)   measuring the incorporation of ^{3}H-thymidine into cells of the culture, wherein the protein has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein.

20.   A pharmaceutical composition comprising:
        (a)   an antibody that (i) immunospecifically binds CD30, (ii) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, and (iii) is not monoclonal antibody AC10 or HeFi-1 and does not result from cleavage of AC10 or HeFi-1 with papain or pepsin, in an amount effective for the treatment or prevention of Hodgkin's Disease; and
        (b)   a pharmaceutically acceptable carrier.

21.   The method of claim 20, wherein the antibody is human, humanized or chimeric.

22.   The method of claim 20, wherein the antibody is conjugated to a cytotoxic

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agent.

23. The method of claim 20, wherein the antibody is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody.

24. The method of claim 20, wherein the cytostatic or cytotoxic effect is determined by:

(a) contacting a culture of the Hodgkin's Disease cell line with the antibody, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;

(b) exposing the culture to 0.5 µCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

(c) measuring the incorporation of ^{3}H-thymidine into cells of the culture, wherein the antibody has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the antibody.

25. A pharmaceutical composition comprising:

(a) a protein, which protein (i) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, (ii) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line, and (iii) is not monoclonal antibody AC10 or HeFi-1 and does not result from cleavage of AC10 or HeFi-1 with papain or pepsin, in an amount effective for the treatment or prevention of Hodgkin's Disease; and

(b) a pharmaceutically acceptable carrier.

26. A pharmaceutical composition comprising:

(a) a protein comprising SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:12, SEQ ID NO:14 or SEQ ID NO:16, which protein (i) immunospecifically binds CD30, and (ii) is not monoclonal antibody AC10 and does not result from cleavage of AC10 with papain or pepsin, in an amount effective for the treatment or prevention of Hodgkin's Disease; and

(b) a pharmaceutically acceptable carrier.

27. A pharmaceutical composition comprising:

(a) a protein comprising SEQ ID NO:20, SEQ ID NO:22, SEQ ID

NO:24, SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32, which protein (i) immunospecifically binds CD30, and (ii) is not monoclonal antibody HeFi-1 and does not result from cleavage of HeFi-1 with papain or pepsin, in an amount effective for the treatment or prevention of Hodgkin's Disease; and

5   (b)   a pharmaceutically acceptable carrier.

28.   A pharmaceutical composition comprising:

(a)   a protein comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID NO:10, which protein (i) immunospecifically binds

10 CD30; and (ii) is not monoclonal antibody AC10 and does not result from cleavage of AC10 with papain or pepsin, in an amount effective for the treatment or prevention of Hodgkin's Disease; and

(b)   a pharmaceutically acceptable carrier.

15   29.   A pharmaceutical composition comprising:

(a)   a protein comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:18 or SEQ ID NO:26, which protein (i) immunospecifically binds CD30; and (ii) is not monoclonal antibody HeFi-1 and does not result from cleavage of HeFi-1 with papain or pepsin, in an amount effective for the treatment or prevention of

20 Hodgkin's Disease; and

(b)   a pharmaceutically acceptable carrier.

30.   The method of any one of claims 25-29, wherein the protein is a human, humanized or chimeric antibody.

25

31.   The pharmaceutical composition of any one of claims 25-29, in which the protein is conjugated to a cytotoxic agent.

32.   The pharmaceutical composition of any one of claims 25-29, in which the

30 protein is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody.

33.   The pharmaceutical composition of any one of claims 25-29, wherein the cytostatic or cytotoxic effect is determined by:

35   (a)   contacting a culture of the Hodgkin's Disease cell line with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said

contacting being for a period of 72 hours;

     (b)   exposing the culture to 0.5 µCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

     (c)   measuring the incorporation of ^{3}H-thymidine into cells of the culture, wherein the protein has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein.

34.   An isolated nucleic acid comprising a nucleotide sequence encoding a protein, which protein (a) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, and (b) exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell.

35.   The isolated nucleic acid of claim 34, wherein the cytostatic or cytotoxic effect is determined by:

     (a)   contacting a culture of the Hodgkin's Disease cell line with the protein, said culture being of about 5,000 cells in a culture area of about 0.33 cm^2, said contacting being for a period of 72 hours;

     (b)   exposing the culture to 0.5 µCi of ^{3}H-thymidine during the final 8 hours of said 72-hour period; and

     (c)   measuring the incorporation of ^{3}H-thymidine into cells of the culture, wherein the protein has a cytostatic or cytotoxic effect on the Hodgkin's Disease cell line if the cells of the culture have reduced ^{3}H-thymidine incorporation compared to cells of the same Hodgkin's Disease cell line cultured under the same conditions but not contacted with the protein.

36.   The isolated nucleic acid of claim 35, wherein the protein is not immobilized.

37.   The isolated nucleic acid of claim 35, wherein the Hodgkin's Disease cell line is L428, L450, HDLM2 or KM-H2.

38.   An isolated nucleic acid comprising one but not both of SEQ ID NO:1 and SEQ ID NO:17, one but not both of SEQ ID NO:3 and SEQ ID NO:19, one but not both of SEQ ID NO:5 and SEQ ID NO:21, one but not both of SEQ ID NO:7 and SEQ ID NO:23, one but not both of SEQ ID NO:9 and SEQ ID NO:25, one but not both of SEQ ID NO:11

and SEQ ID NO:27, one but not both of SEQ ID NO:13 and SEQ ID NO:29, or one but not both of SEQ ID NO:15 and SEQ ID NO:31.

39.   An isolated nucleic acid comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29 or SEQ ID NO:31.

40.   An isolated nucleic acid comprising a nucleotide sequence encoding a protein which comprises one but not both of SEQ ID NO:2 and SEQ ID NO:18, one but not both of SEQ ID NO:4 and SEQ ID NO:20, one but not both of SEQ ID NO:6 and SEQ ID NO:22, one but not both of SEQ ID NO:8 and SEQ ID NO:24, one but not both of SEQ ID NO:10 and SEQ ID NO:26, one but not both of SEQ ID NO:12 and SEQ ID NO:28, one but not both of SEQ ID NO:14 and SEQ ID NO:30, or one but not both of SEQ ID NO:16 and SEQ ID NO:32.

41.   An isolated nucleic acid comprising a nucleotide sequence encoding a protein which comprises SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO:22, SEQ ID NO:24, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32.

42.   The isolated nucleic acid of claim 40 or 41, wherein the protein is an antibody.

43.   The isolated nucleic acid of claim 42 comprising a nucleotide sequence encoding an antibody comprising (a) a variable domain of monoclonal antibody AC10, and (b) a human constant region.

44.   The isolated nucleic acid of claim 42 comprising a nucleotide sequence encoding a protein comprising (a) the complementarity determining regions of a variable domain of monoclonal antibody AC10, and (b) human framework regions.

45.   The isolated nucleic acid of claim 42 comprising a nucleotide sequence encoding an antibody comprising (a) a variable domain of monoclonal antibody HeFi-1, and (b) a human constant region.

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46.   The isolated nucleic acid of claim 42 comprising a nucleotide sequence encoding a protein comprising (a) the complementarity determining regions of a variable domain of monoclonal antibody HeFi-1, and (b) human framework regions.

47.   An isolated nucleic acid comprising a nucleotide sequence encoding a protein comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:2 or SEQ ID NO:10.

48.   An isolated nucleic acid comprising a nucleotide sequence encoding a protein comprising an amino acid sequence that has at least 95% identity to SEQ ID NO:18 or SEQ ID NO:26.

49.   An isolated nucleic acid which hybridizes to the reverse complement of a DNA consisting of a coding DNA sequence encoding a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:10, under highly stringent conditions, which isolated nucleic acid encodes a protein that immunospecifically binds CD30.

50.   An isolated nucleic acid which hybridizes to the reverse complement of a DNA consisting of a coding DNA sequence encoding a protein consisting of an amino acid sequence selected from the group consisting of SEQ ID NO:18 and SEQ ID NO:26, under highly stringent conditions, which isolated nucleic acid encodes a protein that competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1 and exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line.

51.   A recombinant cell containing a recombinant nucleic acid vector comprising a nucleotide sequence encoding a protein, which protein competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1 and exerts a cytostatic or cytotoxic effect on a Hodgkin's Disease cell line.

52.   A recombinant cell containing a recombinant nucleic acid vector comprising SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:25, SEQ ID NO:27, SEQ ID NO:29 or SEQ ID NO:31.

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Abstract of the Disclosure

The present invention relates to methods and compositions for the treatment of Hodgkin's Disease, comprising administering proteins characterized by their ability to bind to CD30, or compete with monoclonal antibodies AC10 or HeFi-1 for binding to

5   CD30, and exert a cytostatic or cytotoxic effect on Hodgkin's Disease cells. Such proteins include derivatives of monoclonal antibodies AC10 and HeFi-1. The proteins of the invention can be human, humanized, or chimeric antibodies; further, they can be conjugated to cytotoxic agents such as chemotherapeutic drugs. The invention further relates to nucleic acids encoding the proteins of the invention. The invention yet further relates to a method

10   for identifying an anti-CD30 antibody useful for the treatment or prevention of Hodgkin's Disease.
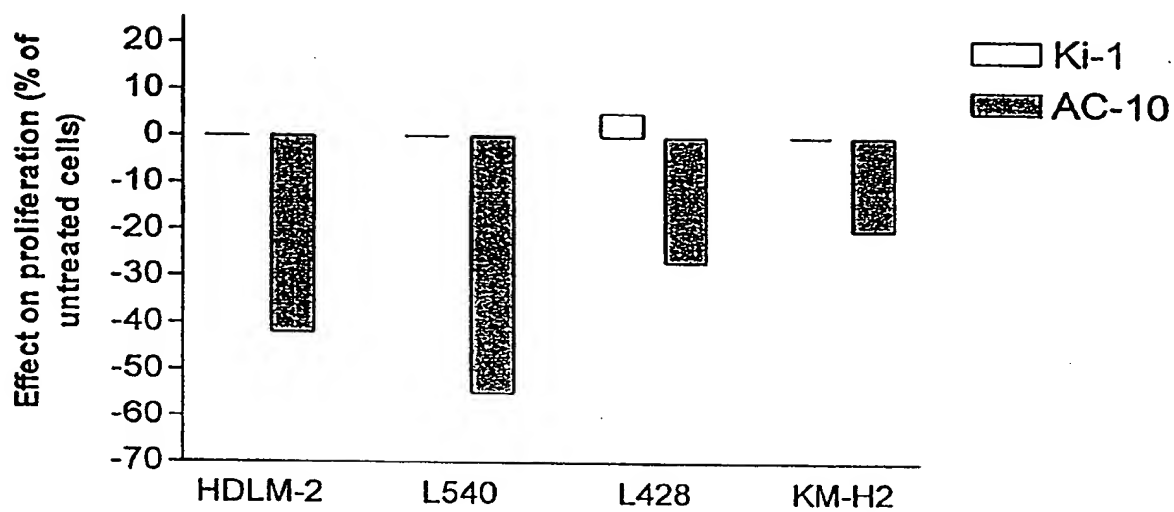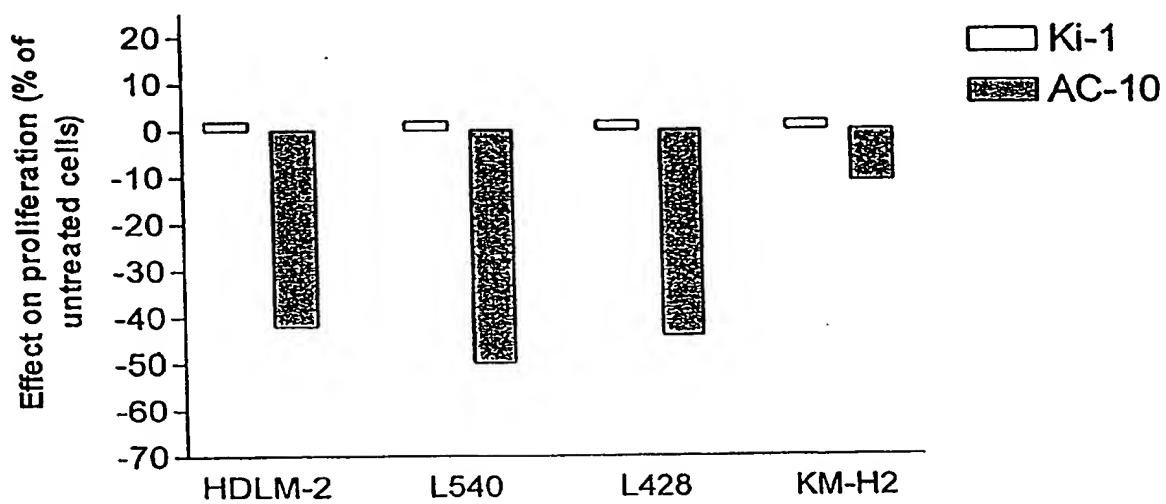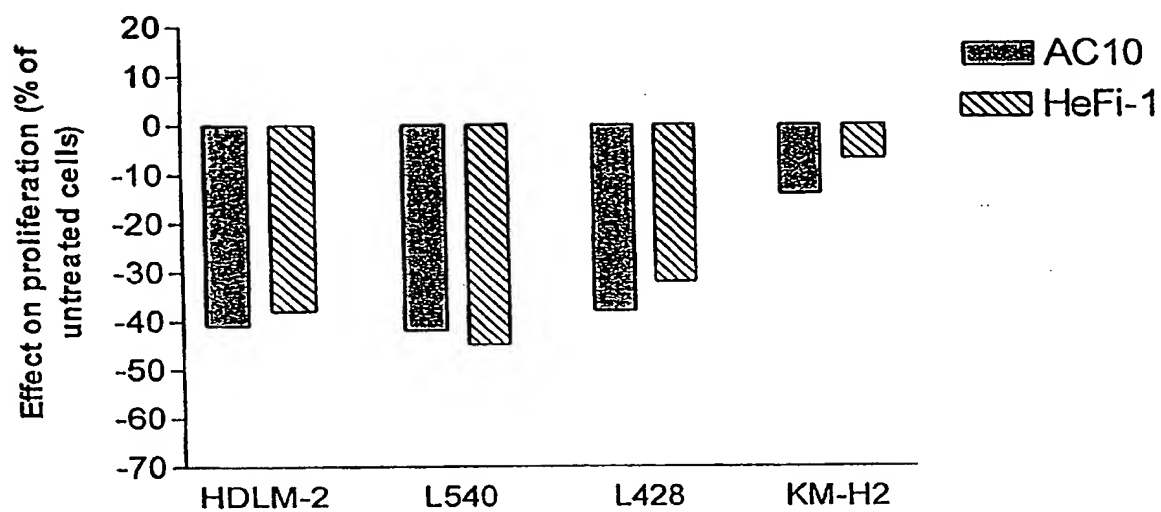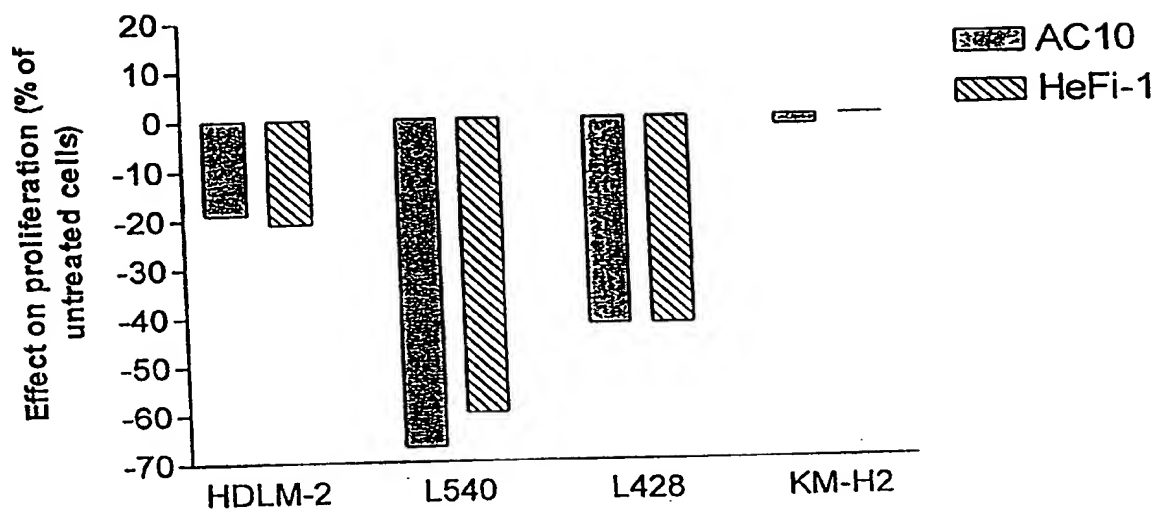
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30

35

FIG. 1

FIG. 2

FIG. 3

FIG. 4
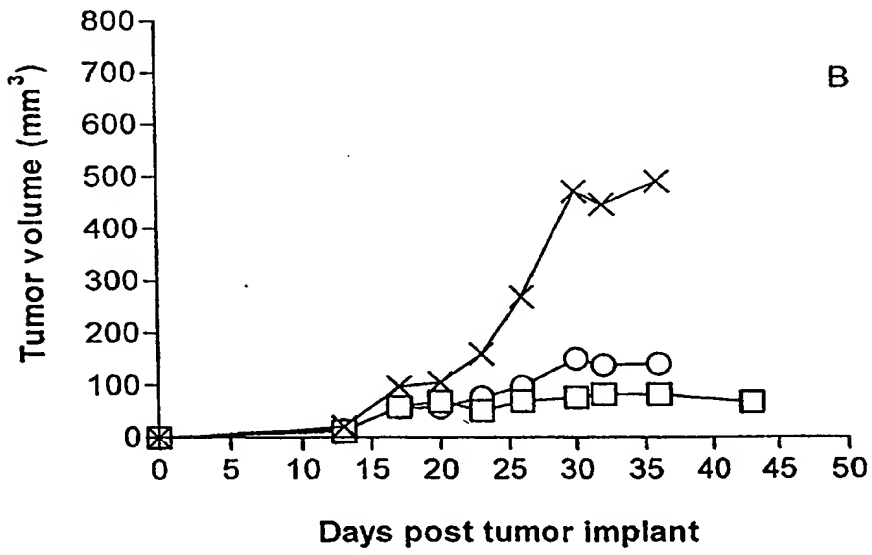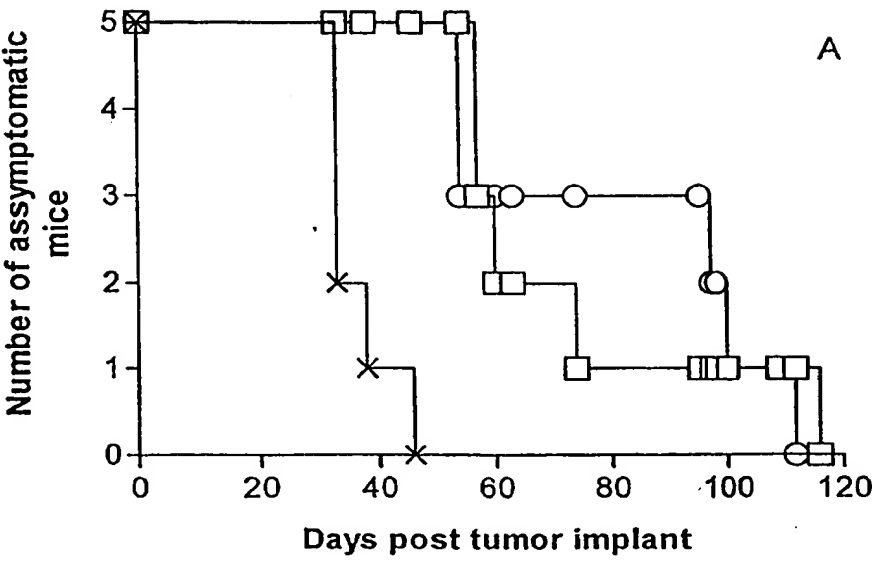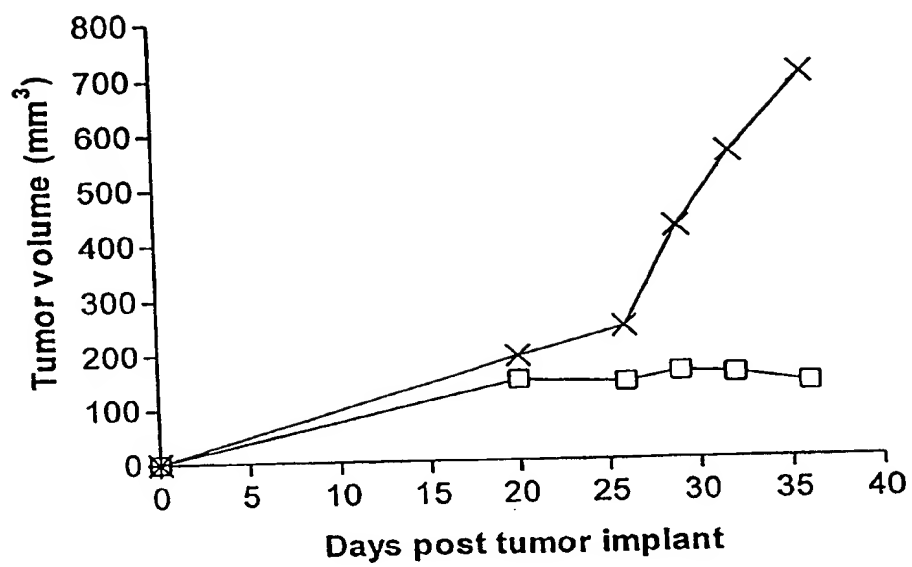
FIG. 5

FIG. 6

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

RECOMBINANT ANTI-CD30 ANTIBODIES AND USES THEREOF

and for which a patent application:
☒ is attached hereto and includes amendment(s) filed on *(if applicable)*
☐ was filed in the United States on as Application No. *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☐ was filed as PCT international Application No. on and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

---

* for use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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2 0 1	FULL NAME OF INVENTOR				
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	POST OFFICE ADDRESS	STREET 21705 92nd Avenue West	CITY Edmonds	STATE OR COUNTRY Washington	ZIP CODE 98020
		SIGNATURE OF INVENTOR 201		DATE	

		LAST NAME Risdon	FIRST NAME Grant	MIDDLE NAME	
2 0 2	FULL NAME OF INVENTOR				
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		SIGNATURE OF INVENTOR 202		DATE	

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		SIGNATURE OF INVENTOR 203		DATE	

		LAST NAME Siegall	FIRST NAME Clay	MIDDLE NAME	
2 0 4	FULL NAME OF INVENTOR				
	RESIDENCE & CITIZENSHIP	CITY Edmonds	STATE OR FOREIGN COUNTRY Washington	COUNTRY OF CITIZENSHIP USA	
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		SIGNATURE OF INVENTOR 204		DATE	

NY2 - 1143163.1

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CD30-SPECIFIC AB1-AB2-AB3 INTERNAL IMAGE ANTIBODY NETWORK: POTENTIAL USE AS ANTI-IDIOTYPE VACCINE AGAINST HODGKIN'S LYMPHOMA

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The tumor-associated CD30 antigen is presently under study as a target for active specific immunotherapy of Hodgkin's lymphoma with anti-idiotypic antibodies. Internal image antibodies (Ab2β) 9G10 and 14G9 against the CD30-specific antibody HRS-4 (Ab1) have been described, which induce a CD30-specific T- and B-cell response in BALB/c mice and New Zealand white rabbits. In extension of this work, murine monoclonal anti-idiotypic Ab2β 9G10, mimicking structures of the nominal CD30 antigen, was used to generate monoclonal Ab3 in mice and polyclonal Ab3 in rabbits with specificity for CD30. The Ab2β 9G10-specific murine monoclonal Ab3 4A4 bound specifically to the 120-kDa band of CD30 present on Hodgkin cell lines and Hodgkin tumor tissue, and effectively inhibited binding of Ab1 HRS-4 to Ab2 9G10 as well as to CD30⁺ cells. Monoclonal Ab3 4A4 was cytotoxic for CD30⁺ cell lines *in vitro* and effectively prevented the s.c. growth of L540 cell tumors after passive i.v. administration in a SCID mouse tumor model. While this cytotoxic effect of the IgM subclass monoclonal Ab3 4A4 was due to complement activation, the murine monoclonal Ab1 HRS-4 and a polyclonal Ab3 preparation of IgG-subclass from New Zealand white rabbits were cytotoxic by an antibody-dependent cell-mediated mechanism *in vitro*. In conclusion, Ab2β 9G10 is able to induce a CD30-specific cytotoxic IgG and IgM response. Cytotoxicity was shown to be mediated by complement activation and antibody-dependent cell-mediated cytotoxicity *in vitro* and *in vivo* and across species barriers. Thus, the CD30-like Ab2β 9G10 may hold promise for effective active specific immunotherapy of human Hodgkin's lymphoma.
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Hodgkin's disease (HD), described in 1832 by Thomas Hodgkin, is a malignancy of the lymphatic system, but its etiology and cell of origin remain unknown. Marked progress in the treatment of this malignancy has been made in the last 20 years and remission can now be achieved by combined modality treatment in more than 90% of all patients. However, 30–40% of all patients will ultimately die from the disease, due to a high rate of relapse (Longo, 1990). The typically low burden of tumor cells compared to an extensive reactive lymphatic infiltrate of the tumor (Stein *et al.*, 1989), the high rate of initially achievable remissions with only low amounts of residual dormant tumor cells remaining and the almost specific expression of the recently cloned CD30-antigen by the tumor cells (Dürkop *et al.*, 1992) may render the use of specific active immunotherapy with tumor-associated antigens a promising approach for the improvement of therapy. Active specific immunotherapy with recombinant CD30-antigen may be hampered by the lack of anti-tumor immunity due to tolerance against tumor antigens (Greene, 1980; Howie and McBride, 1982) as indicated by the presence of soluble CD30 in the sera of patients with HD (Gause *et al.*, 1991). In contrast, the presentation of CD30-like structures buried in the antigenic environment of an anti-idiotypic antibody, as proposed in the network hypothesis of Jerne (1974), may circumvent tumor tolerance. According to this theory, internal image antibodies or Ab2β raised to anti-tumor antibodies (Ab1) mirror the conformation of the original antigen and can induce a protective tumor-specific response in the absence of the nominal antigen. The application of this genetically unrestricted approach has shown encouraging results in experimental systems as well as *in vivo* for the treatment of malignant melanoma and colorectal cancer (Herlyn *et al.*, 1987; Ferrone *et al.*, 1990; Köhler *et al.*, 1989; Levy and Miller, 1990).

In a recent communication (Pohl *et al.*, 1992) we described the induction and characterization of CD30-like Ab2β by the CD30-specific murine monoclonal antibody HRS-4 (Ab1). The murine monoclonal Ab2β 9G10 and 14G9 induced a CD30-specific T- and B-cell response in BALB/c mice and in New Zealand white rabbits. In extension of this work, we describe here the immunogenic characteristics of Ab2β 9G10 by the characterization of a murine monoclonal HRS-4-like Ab3 as well as a polyclonal rabbit Ab3 with specificity for CD30 antigen and their ability to prevent tumor-cell growth *in vitro* and in a SCID mouse tumor model *in vivo*.

MATERIAL AND METHODS

Animals

New Zealand white rabbits and 6- to 8-week-old BALB/c mice were obtained from the Institut fuer Versuchstierzucht, MHH, Hanover, Germany. Animals were maintained on standard chow and water. Pathogen-free mice with severe combined immunodeficiency (SCID) first described by Bosma *et al.* (1983) were obtained from the same institute. Animals were housed and bred in specific-pathogen-free cages and fed autoclaved standard chow and water.

Cell lines

The established CD30-positive human cell lines (L540, L428, L591) were cultured in RPMI 1640 medium (GIBCO, Karlsruhe, Germany), supplemented with 20% (v/v) FCS and glutamine 2 mM/l. Human cell lines HDLM2, P3HR1, 363 (multiple myeloma), L735 and HPB-ALL, BL60 (Diehl *et al.*, 1990), the mouse myeloma cell line X63Ag8.653 (ATCC Rockville, MD) and hybridoma cell lines were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% (v/v) FCS and glutamine 2 mM/l.

Monoclonal antibodies and immunoglobulins

Generation of MAbs HRS-3, HRS-4, anti-POX, 12D3, 9G10 and 14G9 (all IgG$_1$-kappa) used in this study is described elsewhere (Pohl *et al.*, 1992). Myeloma proteins MOPC 41 (lambda chain), RPC 10 (kappa chain) and pooled BALB/c IgG were purchased from Cappel (Veedijk, Belgium), myeloma proteins TEPC 15 (IgA-kappa), MOPC 21 (IgG$_1$-kappa), MOPC 24 (IgG$_1$-lambda), UPC 10 (IgG$_{2a}$-kappa), MOPC 141 (IgG$_{2b}$-kappa), FLOPC 21 (IgG$_3$-kappa), MOPC 37 (IgG$_3$.lambda) and MOPC 104E (IgM-lambda) were from Sigma (Diesenhofen, Germany).

Preparation and labelling of Ig and F(ab')₂ fragments

For ascites production, 2×10^7 viable hybridoma cells were injected i.p. into pristane-primed BALB/c mice. IgG was purified by 2 ammonium sulfate precipitation steps at 50% saturation and subsequent chromatography on a Mono-Q column (Pharmacia, Uppsala, Sweden). Purity of isolated IgG was monitored by SDS-PAGE and F(ab')₂-fragments were prepared by pepsin digestion followed by chromatography on a protein A-Sepharose CL-4B-column (Pohl *et al.*, 1992). Purity of isolated F(ab')₂ fragments was controlled by a sandwich-enzyme-linked immuno-sorbent assay (ELISA) using Fc and F(ab')-specific antibodies and by SDS-PAGE. IgM was purified from ascites by precipitation by dialysis against H_2O for 18 hr at 4°C and subsequent resuspension in 0.1 M NaH_2CO_3 over 72 hr at 4°C. The resuspended IgM-precipitate was IgG-free as measured by a sandwich-ELISA using goat anti-mouse IgM- and IgG-specific antibodies (Dianova, Hamburg, Germany). Biotinylation of purified IgG, IgM or F(ab')₂ fragments was performed as described elsewhere (Pohl *et al.*, 1992) using biotin-amidocaproate-N-hydroxysuccinimide (Sigma). For the induction of Ab3, Ab2 9G10 and 14G9 were coupled to keyhole-limpet hemocyanine (KLH, Calbiochem, Frankfurt, Germany) at a 1 mg/1 mg ratio (Pohl *et al.*, 1992).

Affinity purification of polyclonal rabbit Ab3

Fractions of rabbit IgG were prepared from pooled sera from rabbits immunized with Ab2 9G10 and 14G9 (Pohl *et al.*, 1992). Briefly, after 2 ammonium sulfate precipitation steps, anti-isotypic antibodies were removed from precipitated Ig by subsequent affinity chromatography on mouse-Ig-coated Bio-Gel columns. The unadsorbed fraction was concentrated to the desired volume by ultrafiltration cartridges. For further purification of anti-idiotypic antibodies, 1 mg of anti-isotype-free rabbit IgG in PBS pH 7.4 was processed twice over an Affi-gel 10 column coated with MAb 14G9 or 9G10. The column was then flushed with 100 ml of PBS to elute the unadsorbed fraction. The bound fraction (anti-idiotypic Ig) was eluted with 15 ml 3.5 M ammonium thiocyanate, followed by immediate buffer exchange by chromatography and concentration to the desired volume by ultrafiltration cartridges. The anti-idiotypic Ab3 fraction was essentially IgM-free as measured by a sandwich-ELISA using goat anti-rabbit IgM- and IgG-specific antibodies (Dianova, Hamburg, Germany).

Detection of CD30 antigen by Western blot

Preparation of CD30⁺ cell lysates from L540 cells and CD30⁻ cell lysates from L-735 cell lines has been described in detail (Pohl *et al.*, 1992). The presence of the immunoreactive 120-kDa band of CD30 in the individual L540 batch was confirmed by Western blot analysis and ELISA. CD30⁺ and CD30⁻ samples were separated by SDS-PAGE under reducing conditions in 8% slab gels and separated polypeptides were electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) for Western-blot analysis (Pohl *et al.*, 1992) with the following modifications: After incubation in washing buffer (0.05 M Tris-HCl, pH 9.2) for 10 min, blocking of non-specific binding sites for 1 hr at 20°C with 5% BSA and 0.25% Tween 20 in washing buffer, membranes were incubated with purified monoclonal mouse Ab1 (HRS 4, 10 μg/ml), purified monoclonal mouse Ab3 (4A4, 10 μg/ml) or equal amounts of control antibodies for 1 hr at 20°C in washing buffer. After a washing step (3 × 5 min), membranes were incubated for 90 min at 20°C with biotin-labelled goat anti-mouse-Ig (IgM and IgG specific, Dianova; 1/1,000 dilution). Blots were then washed (3 × 5 min) and incubated with streptavidin-alkaline phosphatase conjugate and alkaline phosphatase substrate solution containing fast red TR salt and naphthol-AS-BI phosphate until a red, insoluble color reaction developed.

Immunization and somatic cell hybridization

Monoclonal Ab2β 9G10 and 14G9 were used for the induction of Ab3 in BALB/c mice and rabbits (Pohl *et al.*, 1992). New Zealand white rabbits were immunized s.c. with 0.5 mg antibody coupled to KLH in complete Freund's adjuvant on day 0, followed by i.m. injections of 0.5 mg antibody-KLH in incomplete adjuvant on days 14 and 28. After subsequent immunization with 0.3 mg of antibody in PBS on days 42 and 56, sera were collected on day 72. The following immunization schedule was used for the generation of monoclonal Ab3 in mice. Female BALB/c mice, 6–8 weeks old, were immunized 6 times over a 72-day period with 100 μg of 9G10-KLH in complete (2×) and incomplete Freund's adjuvant (2×) i.p., and then injected twice with 100 μg Ab2β-KLH in PBS alone. Animals were boosted i.p. with 100 μg of Ab2β in PBS on day 72 and spleen cells were fused 3 days later. Immunization and fusion of spleen cells with the non-secretory X63Ag8.653 mouse myeloma line, using polyethylene glycol (MW 1500, 40% w/v, Boehringer, Mannheim, Germany), has been described (Pohl *et al.*, 1992).

Hybridomas were seeded in 96-well plates and the culture fluids of HAT-resistant hybridomas were tested for anti-Id activity by ELISA 14 to 17 days after fusion. Positive cultures were cloned by limiting dilution and expanded in culture and as ascites tumours in pristane-primed BALB/c mice. Immunoglobulin subclasses of MAbs were determined by an isotyping kit according to the manufacturer's recommendations (Amersham, Braunschweig, Germany).

Screening assay for anti-anti-idiotypic antibodies

Screening for anti-Ab2 activity was performed as follows: ELISA-plates (Greiner, Nürtingen, Germany) were coated with whole IgG or F(ab')₂ fragments of purified 9G10, 14G9 or an unrelated monoclonal anti-POX-antibody of the same isotype and allotype (2 μg/ml, 50 μl/well) overnight, then non-specific protein binding was blocked by 1% gelatin in PBS. Dilutions of immune sera or culture supernatants (50 μl/well) were incubated for 90 min at room temperature, then washed extensively. Biotinylated 9G10 or 14G9 (1 μg/ml, 50 μl/well) was incubated for 1 hr at room temperature and, after extensive washing, a 1/5,000 dilution of alkaline phophatase-conjugated streptavidin (Boehringer) was added for 30 min at room temperature. The reaction product was developed using p-nitrophenyl-phosphate as substrate (Sigma). The degree of substrate conversion was determined at 405 nm using a Titertek ELISA reader (Flow, Irvine, UK).

For further characterization, various myeloma proteins, MAbs of known specificity and subclass (anti-POX), mouse immunoglobulin fractions and normal BALB/c mouse sera were coated at varying dilutions to microtiter plates, and binding of biotinylated antibody was determined as described.

Inhibition assay

To determine whether Ab3 binds at or near the paratope of Ab2, the following inhibition experiments were performed. Ab2β 9G10 was coated to microtiter plates at a concentration of 0.5 μg/ml at 4°C overnight. Non-specific binding was blocked with 1% gelatin. Biotinylated monoclonal Ab1 HRS-4 at non-saturating concentrations (300 ng/ml) was incubated in the presence of varying dilutions of supernatants of monoclonal Ab3 4A4 or relevant control antibodies for 1 hr at room temperature. The assay was processed further as described above. Inhibition of Ab1 binding was calculated as follows:

$$\% \text{ inhibition} = \frac{[1 - (OD_{405} \text{ probe} - OD_{405} \text{ background})]}{(OD_{405} \text{ max} - OD_{405} \text{ background})} \times 100$$

OD_{405} max represents maximal binding of Ab1 without inhibitor, whereas OD_{405} probe represents binding in the presence of an inhibitor. OD_{405} background represents background activity. All assays were performed several times in triplicate. The

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results are presented as the mean value of the assays. The SD of the data was below 10%.

Flow cytometric analysis

Cells of interest were reacted with supernatant or purified antibodies diluted in culture medium in the presence or absence of various inhibitors for 90 min at room temperature. The reaction was developed with goat anti-mouse-$F(ab')_2$-FITC labeled IgG-antibody (Dianova). Flow cytometric analysis was performed on a FACSscan (Becton Dickinson, Heidelberg, Germany) analyzer with the FACSscan software according to the manufacturer's recommendations.

Immunohistology

Cryostat sections were prepared from involved lymph nodes of a patient with Hodgkin's lymphoma (mixed cellularity), fixed with ice-cold acetone, blocked with 1% BSA and incubated with the immunoglobulin of interest diluted in PBS for 90 min at room temperature. Antibody binding was visualized using the APAAP technique as described by Stein *et al.* (1989).

Antibody-dependent cytotoxicity

The antibody-dependent cytotoxicity (ADCC) induced by Ab3 was determined by propidium iodide uptake by avital cells with flow cytometric analysis as described above according to the method of Slezak and Horan (1989) with the following modifications. Vital $CD30^+$ L540 cells were labelled with the fluorescent dye PKH_2 (fluorescence at 480 nm, Sigma, Munich, Germany) and used as targets at a concentration of 1 × 10^4/well. They were incubated with varying amounts of rabbit Ab3-IgG, murine MAb3 4A4 or relevant controls. Peripheral blood lymphocytes purified by Ficoll-gradient centrifugation from several healthy donors were added to the effector cell/antibody mixture at the indicated effector/target-cell ratios. The final culture volume was 200 μl. PKH_2-marked target cells with antibodies and without effector cells served as controls. After 4 hr of culture (37°C, 5% CO_2) the uptake of propidium iodide (2 μg/ml, Sigma) by avital PKH_2+ cells compared to the controls was determined by flow cytometric analysis. The percentage of lysed cells was calculated as:

$$100 \times \frac{\text{experimental uptake} - \text{spontaneous uptake}}{\text{maximal cell number} - \text{spontaneous uptake}}$$

Maximal cell number defines the number of PKH_2-labelled cells per probe. Spontaneous uptake was defined as the number of cells taking up propidium iodide in the absence of effector cells and in the presence of antibody. Experimental uptake was defined as the number of cells taking up propidium iodide in the presence of effector cells and antibody. All assays were performed several times in triplicate. The results are presented as the mean value of the assays. The SD of the data was below 10%.

Complement-mediated cytotoxicity

The complement-dependent cytotoxicity (CDC) induced by Ab3 was determined by propidium iodide uptake by avital cells with flow cytometric analysis as described above, with the following modifications. Freshly drawn mouse or rabbit blood was allowed to clot at 4°C overnight. Serum was collected, pooled, aliquoted, stored frozen at −70°C and used as a source of complement. Complement-free serum was obtained by inactivation of complement activity by incubation of serum samples for 45 min at 56°C. PKH_2-labelled $CD30^+$ (L540) and $CD30^-$ (L735 or HPB-ALL) cells at a concentration of 1 × 10^4/well were used as targets for CDC and incubated with supernatants of murine MAb3 4A4, murine MAb1 HRS-4 or HRS-1 or relevant controls in the presence of propidium iodide at a total volume of 100 μl. Complement was added at a 1/1 ratio to a final volume of 200 μl; target cells with antibodies and without complement served as controls. After 4 hr of culture at 37°C (5% CO_2), propidium iodide was added and

the uptake by PKH_2-labelled cells compared to controls was determined by flow cytometric analysis as described above. All assays were performed several times in triplicate. The results are presented as the mean value of the assays. The SD of the data was below 10%.

In vivo *tumor neutralization studies*

SCID mice were bred as described above. For passive immunization with Ab3, various antibody preparations or mock solutions were administered i.v. in a volume of 200 μl via the tail vein to 4- to 6-week-old female SCID mice in groups of 10, followed by a tumor-cell challenge 1 hr later. For this purpose, a suspension of 1.5 × 10^7 $CD30^+$ (L540CY) or $CD30^-$ (BL60) cells in PBS was injected s.c. at the ventral thoracic wall to induce solid-cell tumors as described by von Kalle *et al.* (1992). Development of tumor size was measured twice weekly with a caliper in a blinded manner, then tumor volume was determined by the formula d × d × D × 3, 14/6 with d as the smaller and D as the larger diameter. Animals were killed when tumor volume exceeded 1 cm^3. Tumors were collected and cryopreserved for further analysis.

RESULTS

Generation of anti-anti-idiotypic antibodies

The CD30-like murine MAb 9G10 of IgG_1-subclass with specificity for Ab1 HRS-4 was used for the induction of syngeneic anti-anti-idiotypic Ab3 in BALB/c mice. KLH-coupled 9G10 induced a humoral immune response with a maximum 56 days after the first immunization. As judged by the lack of detectable binding of the immune sera to an unrelated immunoglobulin of the same isotype, the immune response was directed only against the idiotype of 9G10. The observed immune response was specific for 9G10 and not directed against KLH, as KLH-coupled unrelated MAbs of identical subclass induced no detectable 9G10-specific response (data not shown). After fusion of spleen cells from KLH-9G10-immunized BALB/c mouse 3 with the non-secreting hybridoma cell line X63Ag8.653, hybridomas were screened for anti-anti-idiotypic Ab3 production. Hybridomas which bound to 9G10 as well as to unrelated IgG were considered as anti-isotypic antibodies and not examined further. Because of their binding to 9G10 but not to an unrelated MAb of identical subclass, 6 hybridomas (5 IgM, 1 IgG) were selected initially. Only one Ab3-hybridoma of IgM kappa subclass, termed 4A4, could be stabilized and was cloned for further studies.

Characterization of Ab3 4A4

As shown in Figure 1, monoclonal Ab3 4A4 bound only to $F(ab')_2$ fragments of 9G10 but not to IgG or $F(ab')_2$ fragments from a panel of different antibodies and myeloma proteins of known subclasses. Only a minor cross-reaction with a related
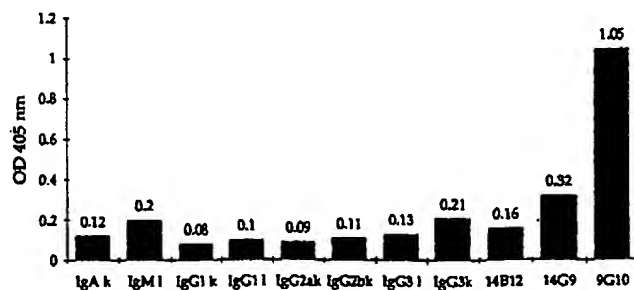
FIGURE 1 – Anti-idiotypic specificities of 4A4 MAb (Ab3) directed against Ab2 9G10. Binding of 0.5 μg/ml of biotinylated anti-anti-idiotypic 4A4 MAb to various MAbs and myeloma proteins ($F(ab')_2$ fragments, 10 μg/ml) was determined by a direct ELISA as described in the text.
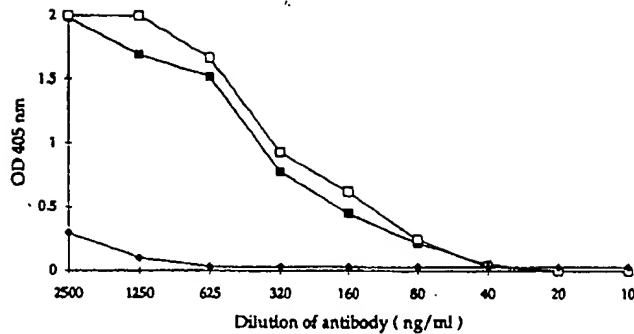
FIGURE 2 – Quantitative binding of murine monoclonal Ab3 to Ab2 F(ab')₂ fragments. Binding of various concentrations of purified and biotinylated monoclonal Ab3 4A4, Ab1 HRS-4 and unrelated control of the same iso- and allotypes was measured with purified F(ab')₂ fragments (0.5 µg/ml) of Ab2 9G10 coated to microtiter plates as described. Results are expressed as mean OD_{405} (n = 3). The SD of the data was below 10%. ■, 4A4-b; □, HRS-4b; ◆, 12D3-b.
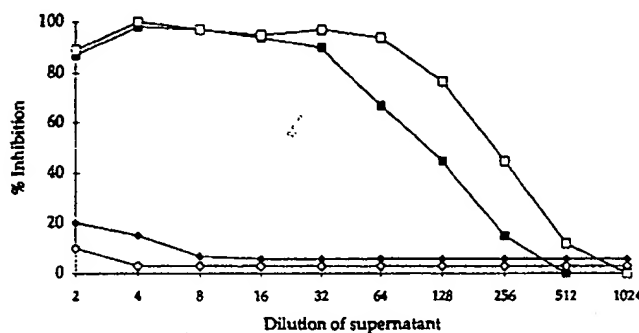

FIGURE 3 – Inhibition of monoclonal Ab1 binding to Ab2 by Ab3. Purified monoclonal Ab2 9G10 was used as plate coat (0.5 µg/ml) and the inhibition of binding of non-saturating amounts (300 ng/ml) of biotinylated monoclonal Ab1 HRS-4 was determined in the presence of varying amounts of unlabeled purified monoclonal Ab3, 4A4, Ab1 HRS-4 and unrelated antibodies of the IgG and IgM subclass. Results are expressed as mean percent inhibition (n = 3). The SD of the data was below 10%. ■, 4A4; □, HRS-4; ◆, 18H10 (IgMk); ◇, POX (IgG1k).

Ab1 HRS-4-specific Ab2β 14G9 was observed. Binding of purified and biotinylated monoclonal Ab3 4A4 to Ab2 9G10 was detectable at concentrations as low as 80 ng/ml, which was comparable to the binding activity of Ab1 HRS-4 to Ab2 9G10 (Fig. 2).

Inhibition of Ab1 binding to Ab2 by Ab3

If Ab3 4A4 were directed at or near the paratope of Ab2, it should inhibit the binding of Ab1 HRS-4. As shown in Figure 3, supernatants of Ab3 4A4 did inhibit the binding of biotinylated HRS-4 to Ab2 9G10 by 100% at a 1/4 dilution and still by 50% at a 1/128 dilution. Comparable inhibition was obtained by unlabeled Ab1 HRS-4, while supernatants from unrelated antibodies of identical subclass gave no relevant inhibition. These data suggest that Ab3 4A4 is directed at the paratope of Ab2 9G10.

Ab-1-like activity of Ab3

To test whether Ab3 represents a true internal image of Ab1 HRS-4 with specificity for CD30, binding with CD30-positive and CD30-negative cell lines was examined by FACS analysis. As shown in Figure 4, Ab3 bound avidly, although to a lesser extent than HRS-4, to the CD30⁺ cell line L540, while no binding to the CD30⁻ cell line HPB-ALL was observed. This binding was specific, since unrelated IgM showed no binding.
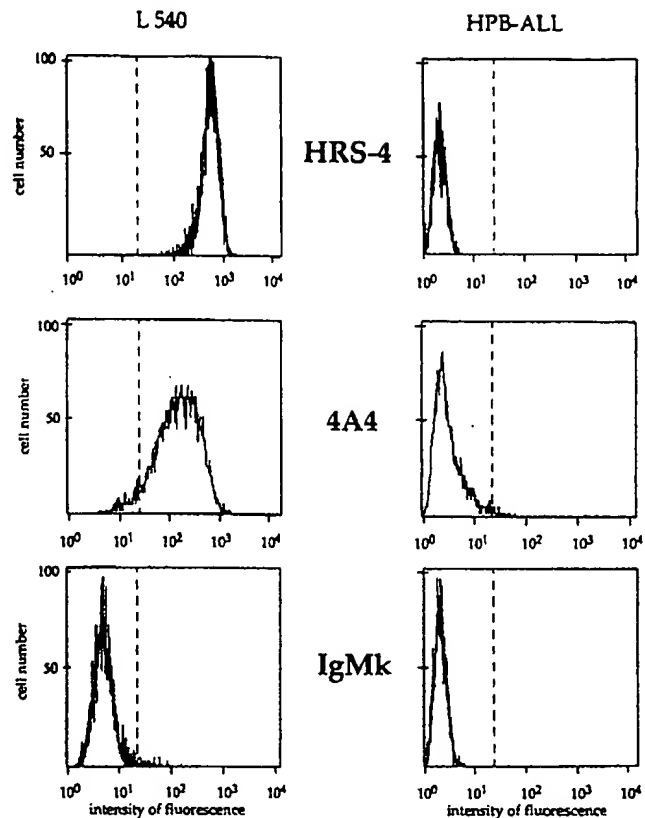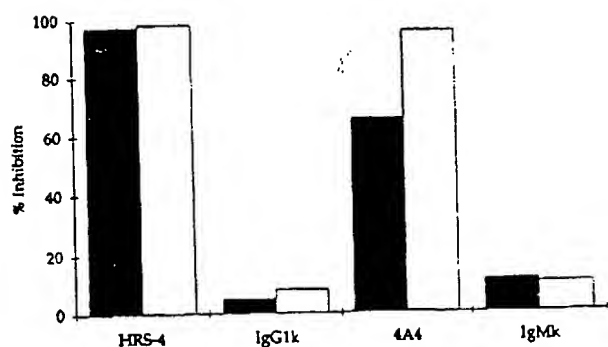

FIGURE 4 – Binding of Ab3 4A4 to CD30⁺ cells. Five hundred thousand CD30⁺ L540 cells or CD30⁻ HPB-ALL cells were incubated with supernatants of monoclonal Ab1 HRS-4, Ab3 4A4 or unrelated IgM for 30 min at 4°C. Antibody binding was detected by FACS with a 1/100 dilution of FITC-labelled goat-anti-mouse Ig (IgM- and IgG-specific).

TABLE I – FLOW CYTOMETRIC ANALYSIS OF BINDING OF MONOCLONAL MURINE Ab1 AND Ab3 TO CD30⁺ AND CD30⁻ CELL LINES

Cell line	HRS-4	4A4	IgM
L540	+	+	−
L428	+	+	−
L591	+	+	−
HDLM2	+	+	−
363(MM)	+	+	−
HPB-ALL	−	−	−
P3HR1	−	−	−
L735	−	−	−

Binding of Ab1 HRS-4, Ab3 4A4 and unrelated IgM to various cell lines was determined as described in the text.

Similar results were found on a variety of CD30⁺ and CD30⁻ cell lines, as summarized in Table I. These data suggested that Ab3 4A4 and Ab1 HRS-4 have a similar binding site to CD30 antigen. This Ab3 should therefore compete with HRS-4 for CD30 binding. As shown by FACS-analysis in Figure 5, binding of biotinylated 4A4 at non-saturating concentrations (1 µg/ml) to the CD30⁺ L540 cell line was inhibited by almost 100% by unlabeled HRS-4. In contrast, Ab3 4A4 inhibited specifically the binding of non-saturating concentrations of biotinylated HRS-4 (0.3 µg/ml) by about 65% at a concentration of 50 µg/ml, indicating a higher binding affinity of HRS-4 than of Ab3 4A4 for CD30. The inhibition of HRS-4 by 4A4 was judged specific since unrelated IgM at equal concentrations induced no inhibition and unlabelled Ab1 and Ab3 inhibited themselves by almost 100%.

FIGURE 5 – Inhibition of Ab1 F(ab')$_2$ fragments to CD30$^+$ cells by murine monoclonal Ab3 4A4. CD30$^+$ L540 cells were incubated with non-saturating amounts of biotinylated Ab1 HRS-4 or Ab3 4A4. The inhibition of binding of a constant, non-saturating amount of F(ab')$_2$ fragments of HRS-4 (0.3 μg/ml) or 4A4 (1.0 μg/ml) in the presence of 50 μg/ml of unlabelled monoclonal Ab3 4A4, unlabelled monoclonal Ab1 HRS-4 and unrelated IgG and IgM of the same isotype was determined with streptavidin-FITC by FACS analysis as described in the text. ■, HRS-4b; □, 4A4-b.

Immunohistology and Western-blot analysis of Ab3 specificity

Further evidence that monoclonal Ab3 4A4 and Ab1 HRS-4 share idiotopes is provided by immunohistochemistry and by Western-blot analysis of antibody binding to CD30$^+$ cell lysates. Ab3 4A4 and Ab1 HRS-4 gave identical staining patterns on cryostat sections of lymph nodes with CD30-positive cells from a patient with Hodgkin's lymphoma (Fig. 6) and a wide variety of malignant and normal tissue (not shown). As shown in Figure 7, Ab1 HRS-4 reacts specifically with a 120-kDa protein and a somewhat smaller degradation product present only in lysates of CD30$^+$ cell lines (Pfreundschuh et al., 1990). Supernatants from murine monoclonal Ab3 4A4 with specificity for Ab2 9G10 reacted in an identical manner with these CD30-specific bands. No such reactivity was seen with lysates from CD30-cell lines or with unrelated IgMk (Fig. 8). These results confirm that Ab3 4A4 shares idiotopes with Ab1 HRS-4 and thus support the internal image nature of the Ab2β 9G10.

Complement-dependent cytotoxicity of Ab3 4A4 in vitro

Thus, having further established that Ab2 9G10 could induce a CD30-specific humoral immune response, it was of interest to evaluate the cytolytic effects of this immune response *in vitro* and *in vivo*. To test complement-mediated cytotoxicity, supernatants of Ab3 4A4, Ab1 HRS-4, affinity-purified polyclonal rabbit anti-9G10-IgG and unrelated IgM were incubated with CD30$^+$ (L540) and CD30$^-$ (HPB-ALL) cells in the presence of activated and inactivated murine or rabbit complement, respectively. As shown in Figure 8, a 28% specific lysis of CD30$^+$ cells was induced by monoclonal IgM-Ab3 4A4, whereas unrelated IgM induced only about 10% lysis. In contrast, the effect of polyclonal rabbit anti-9G10 IgG (13%), and monoclonal Ab1 HRS4 (12%) was not significantly above background activity. The spontaneous rate of cell lysis was 2% (not shown).

Antibody-dependent cell-mediated cytotoxicity

To assess what was the contribution of ADCC and whether human effector cells could mediate cytolysis via Ab3, purified Ab3 4A4 and polyclonal rabbit anti-9G10 IgG were co-incubated with CD30$^+$ (L540) and CD30$^-$ (HPB-ALL) cells as targets in the presence and absence of various amounts of human peripheral blood lymphocytes as effector cells (Fig. 9). Only the polyclonal rabbit anti-9G10 IgG demonstrated a significant CD30-specific cytolytic effect which was dependent on dose and effector-cell number ($p < 0.01$ at a 50/1 effector-cell ratio), while Ab3 4A4 and unrelated murine IgG or IgM

showed only background activity. The spontaneous rate of cytolysis was 3% and no cytolytic effect was observed on the CD30$^-$ cell lines HPB-ALL or L735 (5%, not shown). These data suggest that very little, if any, of the cytolytic activity of IgM-Ab3 4A4 is mediated by ADCC, while polyclonal rabbit IgG-Ab3 exerts a major cytolytic effect via ADCC.

Prevention of tumor growth in vivo by Ab3 4A4

We then tested the ability of Ab3 4A4 to prevent tumor growth *in vivo*. Groups of 10 SCID mice received either 150 μg purified Ab1 HRS-4 or 150 μg Ab3 4A4 or PBS, or an equal amount of unrelated IgG or IgM. To evaluate the contribution of ADCC by the still-functioning natural-killer-cell activity in SCID mice, additional groups of mice receiving Ab1 HRS-4 and Ab3 4A4 were pre-treated with the anti-asialo GM1 MAb. All animals received, 1 hr after passive administration of Ab3, an s.c. injection of a number of L540 tumor cells, which had been titrated to permit palpable tumor growth within 1 week in 100% of otherwise untreated animals.

As shown in Figure 10a, 10 of 10 animals treated with PBS alone, unrelated IgG and unrelated IgM developed a palpable tumor within 15 days after tumor challenge. In contrast, no palpable tumor was detected in the HRS-4- and 4A4-protected animals even 32 days after tumor challenge. At this time the tumor size in the control group exceeded 1 cm^3 and the animals were killed. The differences in tumor growth observed on day 32 were highly significant (4A4 vs. control, $p < 0.01$, HRS-4 vs. control, $p < 0.0001$). Histologic examination of these animals revealed solid tumors of CD30$^+$ cells (not shown). Thereafter, tumor growth in the 4A4-treated group was observed with kinetics comparable to those of the unprotected groups. Histologic examination of these animals revealed solid tumors of CD30$^+$ cells identical to those of the controls (not shown). NK-cell depletion by anti-asialo GM1, as shown in Figure 10b, did not result in tumor growth in 4A4-treated animals. However, NK-cell depletion permitted tumor growth in HRS-4-protected animals and palpable tumors started to develop in the HRS-4-protected, GM1-treated group as early as 15 days after tumor challenge. The tumor growth in this group had kinetics comparable to those of the unprotected groups. Histologic examination of the animals revealed solid tumors of CD30$^+$ cells identical to those of the controls (not shown). When solid CD30$^-$ (BL60) tumors were established, no inhibition of tumor growth was induced by pre-treatment with Ab3 4A4 or control antibodies (not shown). These data indicate that Ab3 4A4 can effectively prevent the growth of solid CD30$^+$ (L540)-cell tumors, mainly due to a cell-independent complement-activation mechanism. In contrast, HRS-4 is most efficient in the prevention of tumor growth due to an anti-asialoglycoprotein-inhibitable, NK-cell-mediated mechanism.

DISCUSSION

The network hypothesis of Jerne (1974) proposes that certain anti-idiotypic antibodies (Ab2β) against antigen-specific antibodies can mimic the biologic effects of nominal antigen and can substitute for this antigen (Köhler et al., 1989). In the search for alternative therapy regimens for Hodgkin's lymphoma, the approach of active specific immunotherapy based on the anti-idiotype vaccine concept may be promising for several reasons: (1) Numerous "internal-image" antibodies have been reported to induce specific immunity against tumor-associated antigen *in vitro* and *in vivo* (Köhler et al., 1989). However, clinical studies with encouraging results, such as those presented by Herlyn et al. (1987) for colon carcinoma and by Ferrone et al. (1990) for malignant melanoma, suffered the handicap of a large tumor burden. Hodgkin's lymphoma, in contrast, is typically composed of relatively few malignant cells compared to an extensive infiltrate of reactive cells, thus considerably lowering the number of malignant cells to be
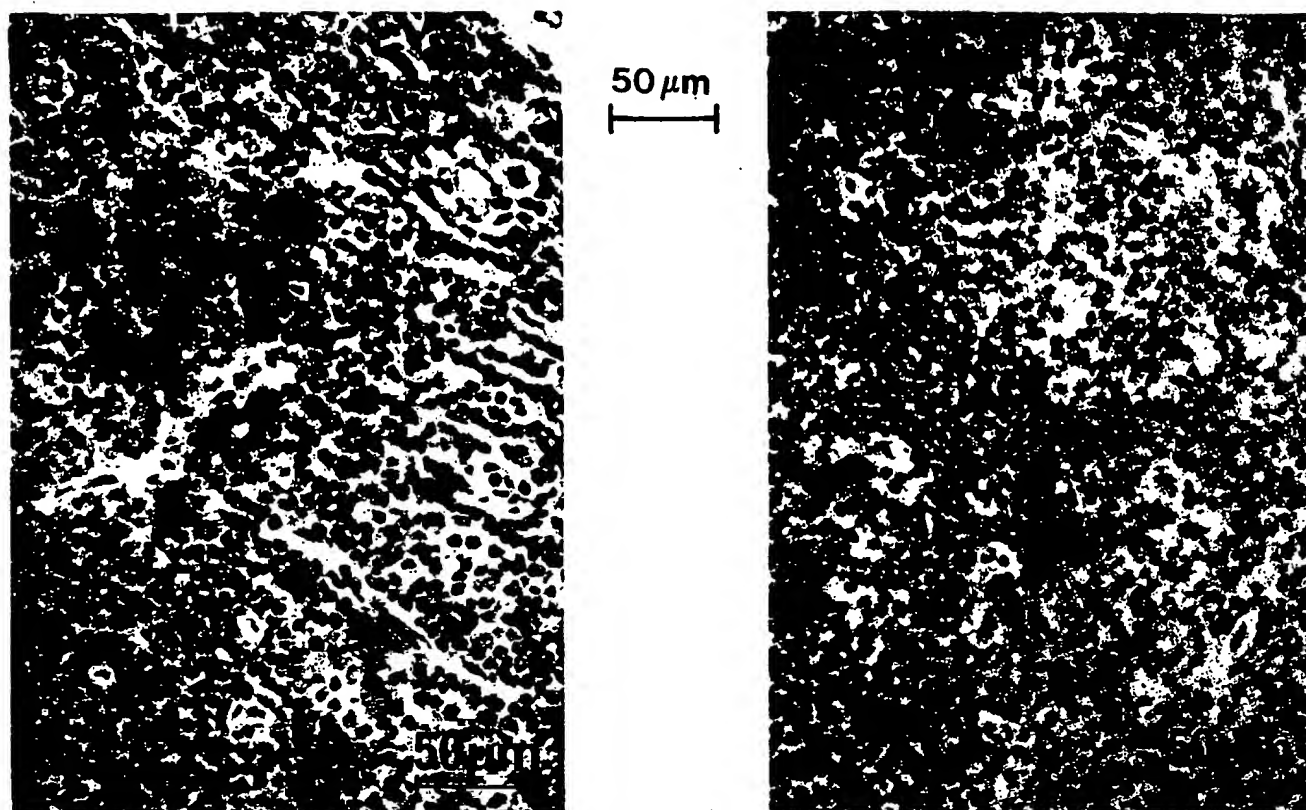
50 μm

FIGURE 6 – Immunohistochemical staining of CD30+ cells. Serial cryostat sections of involved lymph nodes from a Hodgkin patient (mixed cellularity) were stained with (a) Ab1 HRS-4 (2 μg/ml) and (b) purified murine monoclonal Ab3 4A4 (5 μg/ml). Binding was visualized by the immunohistochemical APAAP-technique (see text).

overcome by an immunotherapeutic approach (Stein et al., 1989). (2) A high rate of remissions can be achieved in this disease, with only very low numbers of malignant tumor cells remaining (Longo, 1990). (3) The so-called Ki-1 or CD30 antigen, which is defined by a large panel of specific antibodies, is almost exclusively expressed in Hodgkin's lymphoma. In healthy patients, the antigen is present only in a small subpopulation of lymphocytes in normal tonsils and lymph nodes (Pfreundschuh et al., 1990; Stein et al., 1989) thus representing a rather specific target for immunotherapy. (4) This recently cloned CD30 antigen is thought to be a member of the nerve growth-factor-receptor superfamily (Dürkop et al., 1992). While the open reading frame of the CD30 genome codes for a molecule with a weight of about 64 kDa, the Ki-1 or CD30 antigen is expressed predominantly as a heavily glycosylated 120-kDa membrane-bound protein on the surface of cells of Hodgkin's lymphoma and certain non-Hodgkin lymphomas. In these patients a soluble 88-kDa form of CD30 was described, which disappeared during remission of the disease and recurred in sera of patients with relapse (Gause et al., 1991). The presence of CD30 antigen in the sera of HD patients suggests tolerance towards this tumor antigen (Greene, 1980; Howie and McBride, 1982) and thus would limit the use of purified or recombinant CD30 antigen for active specific immunotherapy. With regard to the prevention of tumor relapse, active immunization with CD30-like structures presented as "network antigen" in the environment of a murine MAb has been shown to enhance T-cell responses and to stimulate "silent clones" in the host, and thus may overcome tolerance towards tumor antigen (Köhler et al., 1989).

To this end, we have described the generation and characterization of syngeneic anti-idiotypic mouse monoclonal Ab2β directed against the CD30 combining site of the HRS-4 antibody and mimicking structures of the nominal CD30

antigen (Pohl et al., 1992). One monoclonal Ab2β 9G10 was shown to induce, in the absence of CD30, a humoral polyclonal murine and rabbit Ab3 response as well as a syngeneic T-cell-mediated immunity that was specific for CD30. In extension of this work, we demonstrate here that a murine syngeneic monoclonal Ab3 4A4, induced by Ab2β 9G10, bound specifically to this antibody and to the nominal antigen CD30 present in cell lysates, on the surface of tumor-cell lines as well as in human tissue as shown by ELISA, flow cytometry, Western-blot analysis and immunhistochemistry. This monoclonal Ab3 shares idiotopes with the Ab1 HRS-4 as it could inhibit effectively binding of Ab1 to CD30 cells. These data suggest that we have established a genuine syngeneic monoclonal Id-cascade, as described first for a T-cell leukemia-associated GP37-specific Id-cascade (Bhattacharya-Chatterjee et al., 1988). The idiotypic cascade described here begins with the Hodgkin-associated CD30 antigen, which is defined by Ab1 HRS-4. This antibody is a member of a panel of CD30-specific MAbs, that had previously been obtained by immunization with the CD30+ Hodgkin-derived cell line L428. Ab2 9G10 of this cascade represents a true internal image (Ab2β) of this nominal antigen, as it induced an Ab1'-like Ab3 even across species barriers in mice and rabbits (Pohl et al., 1992). The induction of Ab3 clones of predominantly IgM subclass with lower affinity for the nominal antigen than the original Ab1 was observed similarly in various idiotypic cascades by others (Percy and Harn, 1988; Bhattacharya-Chatterjee et al., 1990; Viale et al., 1989).

With regard to active immunization with Ab2 9G10, it was of interest to establish the mechanism of action by which an Ab3 might elicit a cytotoxic activity. Active immunization with Ab2 9G10 would require the immune system of the BALB/c mouse to be intact, which in turn would prevent the growth of solid L540 tumors. On the other hand, the SCID mouse, character-
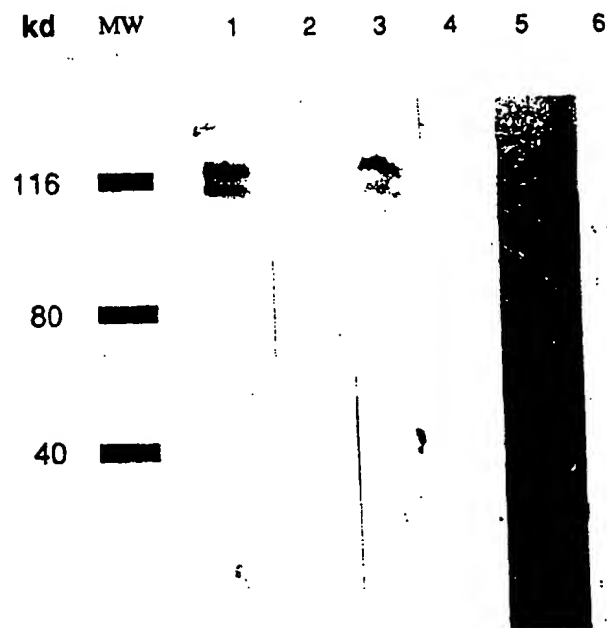
FIGURE 7 – Western-blot analysis of Ab3 4A4-specificity for CD30-antigen. PAGE and Western-blot analysis were performed as described in the text. The molecular weight in kDa is indicated to the left. CD30+ cell lysate from cell line L540 (lanes 1, 3, 5; 50 μl, 1/50 dilution) and CD30− cell lysate from cell line HPB-ALL (lanes 2, 4, 6; 50 μl, 1/50 dilution) were incubated with Ab1 HRS-4 (lanes 1 and 2, 4 μg/ml), purified murine monoclonal Ab3 4A4 (lanes 3 and 4, 10 μg/ml) and with an unrelated IgM-kappa (lanes 5 and 6, 10 μg/ml). Binding was visualized with biotinylated goat anti-mouse antibodies (IgM- and IgG-specific) as described.
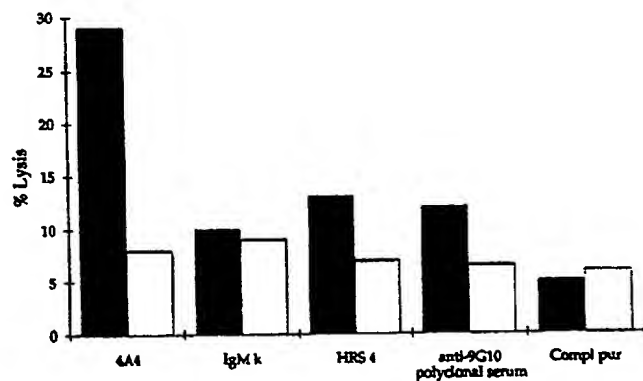
FIGURE 8 – Complement-dependent cytotoxicity induced by Ab3. Supernatants of Ab3 4A4, Ab1 HRS-4, purified rabbit anti-9G10 IgG or unrelated IgM were co-incubated with 1 × 10⁴ PKH₂-labelled CD30+ L540 or CD30− HPB-ALL cells in the presence of active or inactivated murine or rabbit complement, respectively. Cytolysis was measured by the percentage of propidium iodide uptake by PKH₂-labelled cells after a 4-hr incubation at 37°C as described in the text. ■, L540; □, L735.

ized by its T- and B-cell defect which permits growth of solid L540 cell tumors, is, for the same reason, incapable of mounting an effective immune response (von Kalle *et al.*, 1992; Bosma *et al.*, 1983). Lacking thus a valid immunocompetent animal tumor model, we chose to combine the 2 systems and attempted to prevent CD30+ tumor growth by passive administration of the BALB/c derived monoclonal Ab3 to the CD30+ tumor-permissive SCID mouse. To evaluate the contribution of ADCC by the still-functioning natural-killer-cell activity in SCID mice, additional groups of mice receiving Ab1 HRS-4 and Ab3 4A4 were pretreated with the anti-asialo GM1 MAb,
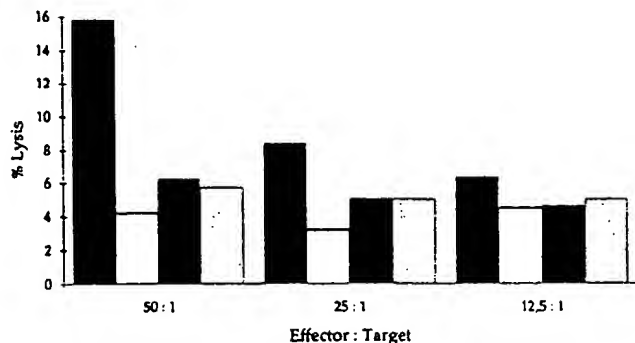

FIGURE 9 – Antibody-dependent cell-mediated cytotoxicity by Ab3. Purified Ab3 4A4, purified rabbit anti-9G10 IgG or unrelated rabbit IgG were co-incubated with 1 × 10⁴ PKH₂-labelled CD30+ L540 or CD30− HPB-ALL cells in the presence of peripheral blood lymphocytes as effector cells in the ratio indicated. Cell-mediated cytolysis was measured by the percentage of propidium iodide uptake by PKH₂-labelled cells after a 4-hr incubation at 37°C (see text). ■, Anti-9G10 polyclonal rabbit IgG (10 μg/ml); □, rabbit IgG (10 μg/ml); ▨, anti-9G10 rabbit polyclonal IgG (1 μg/ml); □, Ab3 4A4 (2 μg/ml).
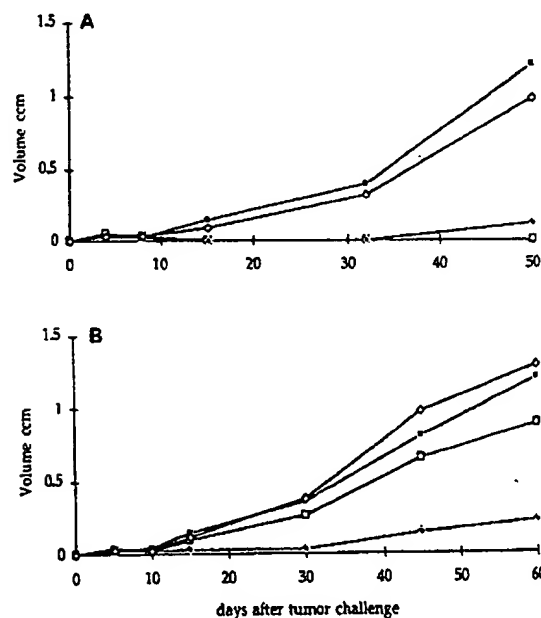

FIGURE 10 – *In vivo* tumor neutralization. *(a)* SCID mice (groups of 10) received i.v. on day 0 either 150 μg Ab3 4A4 or 150 μg unrelated IgM or 150 μg HRS4 or 150 μg IgG₁ kappa in 300 μl PBS or 300 μl PBS alone. One hour after antibody infusion, all animals received 1.5 × 10⁷ L540 tumor cells s.c. The growth of tumors was monitored as described and is presented as the average tumor volume of all tumor-bearing animals per group. Statistically, the observed differences were highly significant. On day 32: 4A4 *vs.* control *p* < 0.01, HRS-4 *vs.* control *p* < 0.0001. *(b)* In addition, 2 groups receiving 150 μg 4A4 or 150 μg HRS-4 were pre-treated i.v. on days −3, 0 and +3 with 100 μg anti-asialo GM1. One hour after infusion of 4A4 or HRS-4 on day 0, all animals received 1.5 × 10⁷ L540 tumor cells s.c. Tumor growth was followed and determined as described. ■, IgG1k; □, HRS-4; ◆, 4A4; ◇, IgMk.

which completely abolishes NK-cell function in mice (Habu *et al.*, 1989). *In vivo* tumor neutralization studies reported here represent a successful prevention of solid Hodgkin cell tumors by "internal-image" antibody-induced monoclonal Ab3 *in vivo*. The exponential growth of tumors in untreated control animals as well as the lack of functioning T and B cells in SCID mice

(Bosma *et al.*, 1983) makes interference by mouse specific T-cell or B-cell-induced tumor-cell lysis most unlikely. Prevention of tumor growth by antibody-dependent cell-mediated cytotoxicity via the residual natural killer-cell activity in SCID mice, however, could be excluded for monoclonal Ab3 4A4. The use of anti-asialo GM1 antibodies, which completely abolish murine NK-cell activity (Habu *et al.*, 1989), had no influence on the protective effect of Ab3 4A4, which was therefore considered to be specific. As further suggested by our *in vitro* data, the protective effect of Ab3 4A4 can thus be ascribed mainly to complement activation. In contrast, Ab1 HRS-4 prevented tumor growth predominantly by ADCC as demonstrated by the reversal of its protective effect after the administration of anti-asialo GM1-antibody. While differences in the affinity and the amount of administered specific antibody binding sites may partly explain the only incomplete effect of Ab3 4A4 on tumor prevention compared to Ab1 HRS, the different mechanism of cytolysis appears to be rather a result of the different isotype than of the idiotype (Davis and Metzger, 1983). The observed ADCC by rabbit polyclonal IgG-Ab3 further supports this assumption. From the above *in vivo* data it appears unlikely that the lack of cell-mediated cytotoxicity by murine Ab3 4A4 is due to the use of allogeneic human effector cells instead of syngeneic murine effector cells. The rabbit-Ab3-mediated lysis of human tumor cells by human effector cells, however, strongly suggests the feasibility of the anti-Id vaccine approach in human subjects.

In summary, the murine monoclonal Ab2β 9G10 has been shown to induce specific B-cell-mediated immunity in both a syngeneic and an allogeneic system and to induce at least syngeneic T-cell immunity (Pohl *et al.*, 1992). Moreover, this antibody induces, depending on the immunoglobulin subclass induced, CD30-specific complement-mediated cytolysis as well as antibody-dependent cellular cytotoxicity, even across species barriers. This immune response was shown to be effective in preventing growth of solid CD30-expressing tumors. While the somewhat anecdotal results obtained by only a single murine monoclonal Ab3 of IgM subclass and a polyclonal IgG preparation of rabbit Ab3 do not allow us to predict the immunogenic effect of Ab2β 9G10 in man, our data clearly indicate that this antibody is a good candidate for idiotypic vaccination against Hodgkin-associated CD30-antigen. Future studies will include histological screening of this antibody on a wide panel of human tissues to establish its safety (*e.g.* to ensure that it will not induce autoimmune disease) and to determine whether it can induce any of the above immune mechanisms in man. The results obtained with our tumor model therefore lead us to propose application of this antibody in a phase-I study in patients with Hodgkin's lymphoma.

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APPENDIX C

In vivo targeting of Hodgkin and Reed-Sternberg cells of Hodgkin's disease with monoclonal antibody Ber-H2 (CD30): immunohistological evidence

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Summary. The ability of the Ber-H2 (CD30) monoclonal antibody (mAb) to target in vivo Hodgkin (H) and Reed-Sternberg (R-S) cells was investigated in six patients with advanced Hodgkin's disease (HD). The patients were injected with scaled-up quantities of 'cold' Ber-H2 mixed-up to a small dose of ^{131}I-labelled Ber-H2, and in vivo binding of the antibody to H and R-S cells was assessed by immunohistological analysis of tumour biopsies and immunoscintigraphy. Only 50% of tumour sites were imaged at scintigraphy by the ^{131}I-labelled Ber-H2. In contrast, immunohistological studies on tissue biopsies, taken 24-72 h following the mAb injection, showed that H and R-S cells in all tumour sites, including those that were not imaged by immunoscintigraphy, were specifically and strongly labelled in vivo by the injected Ber-H2, at a dose as low as 30-50 mg of antibody. In vivo binding of a single dose of Ber-H2 mAb to H and R-S cells did not result in any anti-tumour effect. The excellent in vivo targeting of H and R-S cells with the Ber-H2 mAb may have been the result of multiple favourable factors, including: (a) the restricted expression of the CD30 antigen in normal human tissues; (b) the low level of soluble CD30 in the serum of our patients; and (c) the high affinity of the Ber-H2 mAb for the CD30 molecule. The immunohistological results presented in this study provide a strong argument for using the Ber-H2 mAb as a carrier for delivering cytotoxic agents (isotopes or toxins) to neoplastic cells of HD refractory to conventional therapy.

The Ber-H2 monoclonal antibody (mAb) (Schwarting et al. 1989) recognizes a fixative-resistant epitope of the activation antigen CD30 (Ki-1) (Schwab et al. 1982), a glycoprotein of 120 kD which is strongly expressed on the surface of Hodgkin (H) and Reed-Sternberg (R-S) cells of Hodgkin's disease (HD) (Stein et al. 1985, 1989; Falini et al. 1987) and all cells of a newly recognized category of high-grade non-Hodgkin's lymphoma, i.e. anaplastic large cell (ALC) lymphoma (Stansfeld et al. 1988; Pileri et al. 1989; Falini et al. 1990). In normal human tissues, the Ber-H2 mAb only reacts with a small population of large cells preferentially localized around B-cell follicles (Schwarting et al. 1989).

The restricted in vitro reactivity of Ber-H2 with normal and

pathological human tissues makes it a potential candidate for the immunotherapy of CD30-expressing neoplasms (i.e. HD and ALC lymphomas). However, before it can be proposed for this purpose (especially if the antibody is going to be coupled to cytotoxic agents), it is crucial to know whether the in vivo distribution of the antibody faithfully mimics that observed in vitro. It is, in fact, well known that a number of important factors, including the stability, isotype and molecular size of the mAb, the presence of target antigen in the serum, the size and vascularization of the tumour, the permeability of the vessels to the antibody, and the ability of this antibody to reach its target without being cleared by the liver or spleen, may affect the in vivo distribution of a given mAb (Rosenberg et al. 1989; Schrinberg, 1988).

The purpose of this study was to investigate the in vivo distribution of the Ber-H2 mAb in six patients with advanced

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Table I. Patient data.

Patient (age/sex)	Re-stage*	Diagnosis	Expression of CD30	Dose mAbT (mg)	DoseT (MBq)	Specific activity (MBq/mg)
1. 36/F	IVB (CS)	HD-NS	Strong	0·5	144·3	288·6
2. 52/F	IB₀ (PS)	HD-MC	Strong	15·0	111	7·4
3. 30/F	IIA (CS)	HD-NS‡	Strong	15·0	111	7·4
4. 33/F	IIA (CS)	HD-NS	Strong	30·0	370	12·3
5. 58/M	IVB (CS)	HD-MC	Strong	40·0	185	4·6
6. 32/M	IIIB (CS)	HD-MC	Strong	50·0	nd	nd

CS: clinical staging; PS: pathological staging; HD: Hodgkin's disease; NS: nodular sclerosing; MC: mixed cellularity.
*At the time of relapse. TTotal dose; nd: not done. ‡With areas of lymphocyte depletion HD

PATIENTS AND METHODS

HD. In particular, we focused our attention on answering the following questions: Is the antibody able to reach the tumour cells (H and R-S cells) in a sufficiently high concentration when given intravenously and at which dosage? Is the restricted in vitro reactivity of the Ber-H2 mAb with normal human tissues maintained in vivo?

To achieve this end, patients were injected with scaled-up quantities of 'cold' Ber-H2 mixed with a small dose (1 mg) of ¹³¹I-labelled Ber-H2, and in vivo binding of the antibody to normal and neoplastic tissues assessed by immunohistological studies on tissue biopsies taken 24–72 h following mAb injection and gamma camera immunoscintigraphy.

PATIENTS AND METHODS

Patients. Six patients with histologically confirmed (Lukes, 1971) advanced HD in relapse, who were potential candidates for in vivo immunotherapy with anti-CD30 mAb, were injected with the Ber-H2 mAb (Table I). In all cases, 100% of H and R-S cells detectable in lymph node biopsies taken at initial diagnosis displayed strong surface reactivity with the Ber-H2 mAb. The in vivo binding of the injected antibody to normal and neoplastic tissues was assessed by immunohistological analysis of tumour biopsies and immunoscintigraphy, as described below. The study was approved by the local ethical committee. All patients gave informed consent for the above procedures.

Purification of the Ber-H2 monoclonal antibody. The Ber-H2 mAb (mouse IgG) subclass) was purified from serum-free hybridoma culture supernatant or mouse ascites by affinity chromatography on protein A–sepharose CL-4B, as previously described (Ey et al, 1978). The antibody was eluted with 0·5 M citrate buffer pH 5·0, and dialysed against 0·01 M PBS, pH 7·2. The antibody preparation was >95% pure, as determined by SDS-PAGE analysis. After ultracentrifugation to remove microaggregates, the preparation was passed through a 0·2 μm filter (Millipore) to ensure sterility, and stored at −70°C prior to use. The purified antibody passed the safety control tests as established by the document entitled 'Empfehlungen für die Herstellung und Prüfung in vivo applizierbarer monoklonaler Antikörper' (1987). In particular, the antibody preparation was shown to be free from bacteria, fungi and adventitious viruses (as documented

by the mouse antibody production, MAP, test). The Limulus Amoebocyte Lysate assay for endotoxin was negative. The total content of DNA for dose (15–50 mg) was about 10 picogrammes.

Radiolabelling of Ber-H2. All radiolabelling procedures were performed at the Radio-Pharmaceutical Division of Sorin Biomedica, Saluggia (VC), Italy. The Ber-H2 mAb was radioiodinated using the Iodogen method (Pierce Rockford, Ill.) (Fraker & Speck, 1978). The antibody (2 mg in 0·33 ml PBS at pH 7·4) was added to a reaction vial previously coated with 100 μg Iodogen. Na ¹³¹I (8 mCi:296 MBq) was added to the vial and the reaction mixture incubated for 15 min at room temperature under magnetic stirring. The radiolabelling efficiency was assayed by ascending chromatography on silica gel plate (eluent methanol/water 85:15). The mean labelling yield was >95%. The unincorporated ¹³¹I was separated by anion exchange chromatography through a syringe containing 1–2 ml Dowex AG 1XB resin (Biorad) and eluted with 3 ml of 0·3% HSA 0·04 M phosphate buffer at pH 7·4. After iodination, the Ber-H2 mAb retained more than 70% of its baseline immunoreactivity, as shown by a frozen section assay. The final product was sterile and free of pirogens.

Antibody administration. An intradermal hypersensitivity test was performed in each patient 1 h before mAb administration with 20 μl of 1 μg/ml of the antibody preparation in sterile buffered saline. The site of injection was observed for 15 min. No positive reaction was seen. To minimize thyroid uptake of radioiodine, patients were treated with a saturated solution of potassium iodide (10 drops, three times per day) from 2 d before injection until 10 d after (Rosen et al, 1987). Patient 1 received only 0·5 mg ¹³¹I-labelled Ber-H2. Studies of scaled-up doses were performed by co-injecting patients 2, 3, 4 and 5 with 1 mg ¹³¹I-labelled Ber-H2 and 14 mg (two cases), 29 mg, or 39 mg unlabelled Ber-H2 mAb for a total dose of 15 mg, 30 mg and 40 mg respectively. Patient 6 received only 50 mg of unlabelled Ber-H2. The 0·5 mg dose of radiolabelled Ber-H2 was injected over 10 min. Higher doses of the antibody were given as a continuous 3 h infusion in 500 ml of normal saline containing 5% human serum albumin. Patients were routinely premedicated with 10 mg of clorfeniramine 30 min before antibody administration. Vital signs were recorded frequently during antibody infusion and

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Table II. Correlation between clinical staging, imaging, histology and immunohistology in six HD patients injected with the Ber-H2 (CD30) mAb.

Patient	Clinical staging	Imaging (time)* (h)	Studies post-Ber-H2 injection		
			Histology (% H & R-S)	APAAP	Serum CD30
Case 1					
LN abdomen	+ (CT)	+ (72)	na	na	+ve U/l
Lung	+ (CT)	− (72)	na	na	
Spleen	− (CT)	+ (72)	na	na	
Liver	− (CT)	+ (72)	na	na	
Case 2					
LN abdomen	− (CT)	− (72)	HD-MC (~5%)‡	+	N.D
Spleen	S (CT)	+ (72)	HD-MC (~5%)‡	+	
Liver	− (CT)	+ (72)	Normal‡	−	
BM	−	− (72)	Normal‡	−	
Case 3					
LN neck	+ (CE)	− (72)	HD-NS (~30%)	·	1200 U/l
LN med	+ (CT)	− (72)	na	na	
Spleen	− (CT)	+ (72)	na	na	
Liver	− (CT)	+ (72)	na	na	
Case 4					
LN neck	+ (CE)	+ (48)	HD-NS (~15%)	+	N.D.
LN med.	+ (CT)	E (48)	na	na	
Spleen	− (CT)	+ (48)	na	na	
Liver	− (CT)	+ (48)	na	na	
BM	−	− (48)	na.	na	
Case 5					
LN neck	+ (CE)	− (24)	HD-MC (~5%)	+	100 U/l
LN med	+ (CT)	E (72)	na	na	
LN paraaort.	+ (CT)	+ (72)	na	na	
LN iliac	+ (CT)	+ (72)	na	na	
Spleen	− (CT)	+ (72)	na	na	
Liver	− (CT)	+ (72)	na	na	
BM	−	− (72)	HD-MC	+	
Skin§		− (72)	Normal	−	
Adipose tissue§		− (72)	Normal	−	
Case 6					
LN neck	+ (CE)	na	HD-MC (~5%)	+	na
LN med	+ (CT)	na	na	na	
LN paraaort.	+ (CT)	na	na	na	

LN: lymph node; BM: bone marrow; S: splenomegaly; CE: clinical examination; CT: computerized tomography; med.: mediastinal; H & R-S: Hodgkin and Reed-Sternberg cells; HD: Hodgkin's disease; NS: nodular sclerosing; MC: mixed cellularity; na: not available; E: equivocal image due to high blood background; N.D.: not detectable.

*Refers to the optimal imaging time post-injection of Ber-H2

‡Refers to endogenously bound Ber-H2 to H & R-S cells after in vivo injection of the antibody. Staining was performed on frozen sections as indicated in Materials and Methods.

‡Taken at exploratory laparotomy.

§Adjacent to the biopsied neck LN

the following 12 h. No hypersensitivity reaction occurred. Complete laboratory investigations were repeated on days 1 and 7 following infusion.

Tissue handling. Tumour biopsy was not available in patient 1. Patient 2 underwent a re-staging exploratory laparotomy 72 h following infusion of the Ber-H2 antibody. Intraopera-

tively, the spleen and several abdominal lymph nodes were removed; wedge liver biopsies and a new Jamshidi bone marrow biopsy were also performed (Table II). Patients 3, 4, 5 and 6 had tumour biopsies performed 24-48 h following injection of mAb.

Tissue samples were obtained fresh and split, one portion

being fixed in BS and paraffin-embedded for routine diagnosis and the other snap-frozen in liquid nitrogen, cut at 5 μm to a cryostat and used for immunohistological studies.

Immunohistological studies. Immunohistological analysis was performed on frozen tissue sections that had been previously air-dried overnight at room temperature and fixed in acetone for 10 min (Falini et al. 1984, 1989).

In vivo binding of the Ber-H2 mAb to H and R-S cells in tissue specimens removed following the mAb injection was assessed by direct incubation of tissue frozen sections with the secondary rabbit anti-mouse Ig followed by APAAP complexes (Cordell et al. 1984). In order to avoid any cross-reactivity with human immunoglobulins, the secondary rabbit antibody was pre-incubated with 10% normal human serum 1 h before the immunostaining procedure. Frozen sections from pre-mAb infusion lymph node biopsies incubated with the secondary rabbit anti-mouse Ig antibody served as a negative control, and frozen sections from pre- and post-mAb infusion lymph node biopsies incubated with Ber-H2 as a positive control. Other controls included APAAP staining of post-mAb infusion biopsies with the following mAbs (Knapp et al. 1989), CD3 (UCHT1), CD20 (L26), Ber-Mac 3 (anti-macrophage), and Ki-67 (anti-proliferating cells) (Gerdes et al. 1983).

Radioimmunodiagnostic studies. Whole body multiple anterior and posterior images (4 min per image) were obtained within 2 h and daily for up to 6 d after the mAb infusion using a large-field-of-view gamma scintillation camera (GE maxicamera/37) with a high energy collimator (400 keV maximum). Patient 1 was also subjected to single photon emission computerized tomography (SPECT) 48 and 72 h post-infusion. The data were stored and analysed in an interfaced computer system (MEDUSA, Sopa) which generated digital images. Scans were read independently by two nuclear medical physicians with no prior knowledge of disease sites.

Serum assays. Pre-infusion serum levels of soluble CD30 molecule in the serum were determined in five patients by an ELISA technique, as previously described (Pizzolo et al. 1990) using a prototype kit developed by J. Kyhse-Andersen, Dako a/s, Denmark.

RESULTS

Histological and immunohistological studies

No tissue specimens were available for study from patient 1. All morphologically recognizable H and R-S cells present in tumour sites (superficial and abdominal lymph nodes, spleen and bone marrow) from four out of the five HD patients (Table II; cases 2, 4, 5 and 6), including those encased by bundles of fibrous tissues in cases of nodular sclerosing HD, were specifically labelled in vivo by the injected Ber-H2 mAb (Figs 1 and 2). In cases 4, 5 and 6, APAAP staining of control frozen sections with Ber-H2 mAb gave an identical reactivity pattern, suggesting that most, if not all, CD30 binding sites of neoplastic cells were saturated in vivo by Ber-H2 at dosage of 30–50 mg. Weaker in vivo targeting of tumour cells was observed in patient 2 who received only 15 mg of Ber-H2. No in vivo labelling of neoplastic cells by Ber-H2 was observed in patient 3 (injected with 15 mg mAb). In all positive cases, in

vivo labelling was highly selective, since no other cell constituents but H and R-S cells were stained by the secondary antibody alone in frozen sections from all samples studied. As expected, incubation of frozen sections with a number of primary mAbs other than Ber-H2, resulted in APAAP labelling of both H and R-S cells (due to binding of Ber-H2 in vivo) and the cell population identified by the corresponding mAb in vitro (e.g. macrophages by Ber-Mac3). Hodgkin and R-S cells in pre-mAb infusion lymph node specimens failed to stain with secondary antibody alone and served as a negative control (Fig. 2c).

In spite of positive imaging, the liver of patient 2 was not histologically involved by HD and no Ber-H2 was detected in the organ at immunohistological level. In consequence, the exact localization of radiolabelled Ber-H2 in the liver could not be established.

Radioimmunodiagnostic studies

The clinical characteristics of the six patients studied are listed together with the specific organ sites imaged and the immunohistological results in Table II.

Tumour sites were usually visualized 24 h following mAb injection. However, optimal images were seen at 48–72 h, when blood background activity had decreased. Only 50% of sites previously documented to be clinically or pathologically involved by HD were visualized by immunoscintigraphy with radiolabelled Ber-H2 (Table II; Figs 3 and 4). The best imaging at planar scintigraphy was that seen in patient 4 (Fig. 3) who had large lymph nodes (2–3 cm in size) containing a high percentage of H and R-S cells (~15%). Immunohistological studies (see Fig 2a, b) provided conclusive evidence that the positive imaging in this case was the result of the specific in vivo binding of ^{131}I-labelled Ber-H2 to H and R-S cells rather than the consequence of non-specific uptake of the antibody by cells of the reticulo-endothelial system. This is further supported by the observation that no targeting of neck nodes was observed in this patient following injection with ^{131}I-labelled unrelated mAb (anti-CEA) of the same subclass as Ber-H2.

Correlations between imaging and in vivo immunohistological binding were available in cases 2, 3, 4 and 5 (Table II). Noteworthy, lymph nodes from two patients (Table II; cases 2 and 5) were not imaged by ^{131}I-labelled Ber-H2 in spite of the strong in vivo labelling of H and R-S cells by Ber-H2 at immunohistological level.

Non-specific liver and spleen uptake was seen in all patients. Thyroid and urinary bladder uptake of ^{131}I-labelled Ber-H2 was also a common finding, that indicated in vivo dehalogenation. Thyroid uptake occurred in spite of the administration of potassium iodine (Lugol) solution, but did not cause hypothyroidism in any of our patients.

Clinical effects and toxicity

There was no measurable decrease in the size of tumour sites during the interval (range 15–40 d) between mAb infusion and beginning of salvage therapy in any of our patients. No toxic effects or laboratory abnormalities were documented for any of the Ber-H2 doses given.
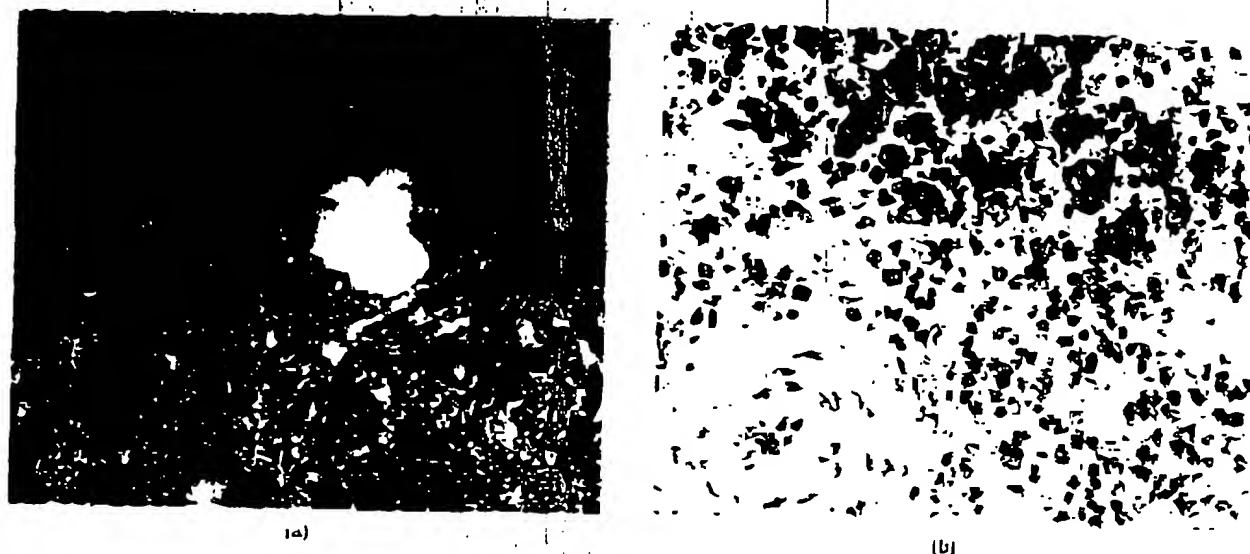
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(a)

(b)

Fig 1. Patient 2 (a) The spleen removed at laparotomy 72 h following mAb injection shows massive involvement by HD. (b) Frozen section from the spleen nodules shown in Fig 1(a) following incubation with rabbit anti-mouse antiserum and detection by the APAAP technique. Notice the selective and strong in vivo binding of the Ber-H2 mAb to neoplastic cells (labelled in red). *Indicates a central arteriole (haematoxylin counterstain. × 400)

Serum assays

Pre-treatment CD30 serum levels in five HD patients are shown in Table II. Detectable levels of soluble CD30 were absent in the serum of 25 healthy controls (Pizzolo et al. 1990).

DISCUSSION

This paper contains the following interesting data: (a) immunohistological evidence that good in vivo targeting of H and R-S cells in all sites involved by HD can be achieved with doses of Ber-H2 mAb as low as 30–50 mg. (b) immuno-

(a)

(b)

(c)
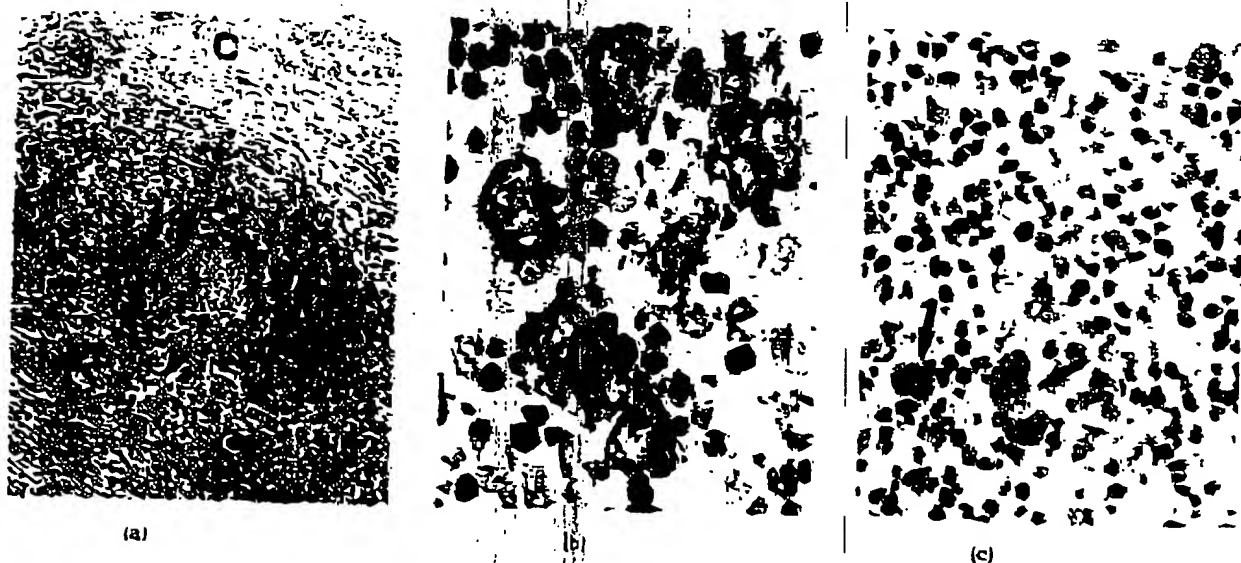
Figs 2a and 2b. Left neck lymph node biopsy taken 48 h following Ber-H2 infusion (patient 4). (a) The frozen section, incubated with rabbit anti-mouse antiserum and developed by the APAAP technique, shows clusters of H and R-S cells (labelled in red) encased by fibrous tissue. Notice the thickened node capsule (C) (haematoxylin counterstain; × 125). (b) Higher power view of the same field showing strong surface in vivo targeting of H and R-S cells (haematoxylin counterstain; × 800) Fig 2c. No labelling of tumour cells (arrows) is observed in the pre-mAb infusion lymph node biopsy incubated with rabbit anti-mouse antiserum and APAAP complexes (haematoxylin counterstain; × 800).

Fig 3. Patient 4 Anterior view of the head 40 h following infusion of ^{131}I-labelled Ber-H2. A left neck lymphoma mass (T. arrow) is clearly imaged. *Indicates thyroid uptake

histological demonstration of *in vivo* saturation of CD30 antigen sites on target cells not detected by gamma imaging with ^{131}I-labelled Ber-H2; (c) immunohistological evidence that *in vivo* injected Ber-H2 mAb binds exclusively to H and R-S cells, which is entirely consistent with the Ber-H2 staining pattern seen with *in vitro* labelling of tissue sections; (d) optimal *in vivo* binding of a single dose of unmodified Ber-H2 to H and R-S cells does not produce any anti-tumour effect; (e) there are no side-effects associated with the administration of the Ber-H2 mAb at the dosage used in this study.

The most important finding in this study was the immuno-

histological demonstration that, when injected *in vivo*, Ber-H2 is able to reach neoplastic cells in all body sites involved by HD and binds only to H and R-S cells. The previous finding that in paraffin sections R-S cells contain passively absorbed polyclonal, plasma immunoglobulins (Papadimitriou *et al.* 1978) raised the question as to whether the labelling of our patients' H and R-S cells was the result of non-specific *in vivo* binding of the injected Ber-H2 mAb through the Fc fragment rather than through the specific antibody determinant (CD30). However, this possibility was excluded because the binding of the Ber-H2 *in vivo* was identical to that produced by the antibody *in vitro* (e.g. surface staining of all H and R-S cells), whereas human plasma immunoglobulins are detectable within R-S but not H cells and the staining is intracytoplasmic (Papadimitriou *et al.* 1978) Moreover. there was no non-specific *in vivo* binding of Ber-H2 by normal cells bearing the Fc receptors (e.g. macrophages, interdigitating reticulum cells, etc.) in any of the human lympho-haemopoietic tissues studied (lymph nodes, liver, spleen and bone marrow). This finding is in keeping with the immunostaining pattern already described for the Ber-H2 mAb *in vitro* (Schwarting *et al.* 1989) and may be related to the low affinity of the Ber-H2 mAb (mouse IgG1 subclass) for the human Fc receptor. In this regard, the Ber-H2 mAb differs from the prototype anti-CD30 mAb Ki-1 (mouse IgG3 subclass) that has been reported to react with activated macrophages (Andreesen *et al.* 1989).

In vivo targeting of H and R-S cells in our patients occurred under conditions considered to negatively influence the biodistribution of mAbs within neoplastic tissues, e.g. 'bulky disease' and areas of necrosis in the spleen (case 2), and nodular sclerosing histology (case 4). There was immunohistological evidence of *in vivo* binding of Ber-H2 to H and R-S cells from the 24th hour (cases 5 and 6) but it could have
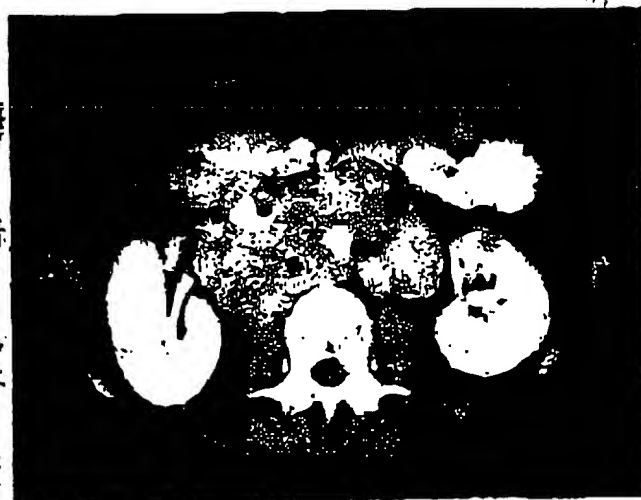
(a)

(b)

Fig 4. Patient 1 (a) The abdominal CT scan shows large lymph node masses (*) between the kidneys. (b) SPECT (single photon emission computerized tomography) taken at the same abdominal level as Fig 4(a), 72 h after mAb infusion. Lymph node masses (T. arrow) are imaged by ^{131}I-labelled Ber-H2.

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occurred even earlier. The antibody was still detectable on tumour cells 72 h post-infusion (case 2).

Noteworthy, a dose of only 30–50 mg of Ber-H2 mAb was sufficient to produce a good in vivo targeting of H and R-S cells in all body sites involved by the disease. This dose is far lower than that reported to be necessary for good in vivo targeting of human lymphomas with other mAbs (Müller et al. 1982; Brown et al. 1989; Press et al. 1987, 1989; Eary et al. 1990). In particular, doses ranging from 500 mg to 1.5 g of anti-idiotype (Brown et al. 1989), IF5 (anti-CD20) (Press et al. 1987) or MB-1 (anti-CD37) (Press et al. 1989) mAbs were found to be necessary for good in vivo labelling of malignant cells in non-Hodgkin lymphomas of B cell type and, despite the high dose, antigenic sites were only partially saturated.

The restricted expression of the CD30 molecule in normal human tissues (a small subset of large cells around B cell follicles) (Schwab et al. 1982; Schwarting et al. 1989) and/or the low level of soluble CD30 molecule detectable in the serum of our patients (unable to saturate the amount of the injected mAb) may have been crucial in favouring the distribution of the Ber-H2 mAb into tumour sites. Additional favourable factors possibly include the high affinity of Ber-H2 for the CD30 molecule; this antibody is directed against a fixative-resistant epitope of the CD30 molecule and has proved to have in vitro a higher avidity for CD30-bearing normal and neoplastic cells than the previously reported mAb Ki-1 (Schwarting et al. 1989). Also the number of neoplastic cells that need to be saturated by the injected mAb in HD is low as compared to non-Hodgkin lymphomas, since H and R-S cells only account for a small percentage of the whole cell population in HD involved tissues.

The lack of immunohistologically documented in vivo binding of Ber-H2 to H and R-S cells in patient 3 may have been due to one or more of the following: low dose of injected mAb (15 mg), mediastinal 'bulky disease' areas of lymphocyte depletion HD, high level of CD30 circulating in the serum (~1200 U/l), anatomical factors of the tumour.

Mixing of a scaled-up dose of 'cold' antibody with a small amount of ^{131}I-labelled Ber-H2 gave us the opportunity to ascertain whether the immunohistologically documented binding of radiolabelled antibody to H and R-S cells was associated with any imaging of tumour sites at planar scintigraphy. Antibody imaging of HD would be of great value in staging of this condition and in predicting targeting of tumour cells by Ber-H2 in patients who are candidates for CD30 mAb immunotherapy. In spite of the documented immunohistological targeting of tumour cells by Ber-H2, only 50% of the tumour sites were imaged at planar scintigraphy. These false negative results may have been due to the small size of the involved lymph nodes (about 1.5 cm in patients 2 and 5) (Murray et al. 1985), and/or the low percentage of H and R-S cells present in the tumour sites (≈5% of the whole cell population). Thus, no conclusion as to the value of ^{131}I-labelled Ber-H2 in imaging of HD can be drawn from this preliminary study and further investigations on a larger series of HD patients, possibly using Ber-H2 labelled with Indium-111 (Carrasquillo et al. 1987) instead of ^{131}I, are warranted.

Our immunohistological demonstration of Ber-H2 antibody binding to all tumour sites, even those that were not

detected by gamma imaging, provides a strong argument for using Ber-H2 as a carrier for delivering cytotoxic agents to CD30-expressing neoplasms (i.e. HD and ALC lymphomas) refractory to conventional therapy. Because HD is highly radiosensitive, conjugates of the Ber-H2 mAb with either ^{131}I or Yttrium-90 (Deshpande et al. 1990) are potential candidates for radioimmunotherapy. CD30-immunotoxins (Ber-H2 labelled to ricin A chain or saporin) should also be considered for immunotherapy of HD, since they exert potent killing activity on cultured HD cell lines (L-428, Colo) in vitro (Engert et al. 1990; Tazzari et al. 1992). Then, the APAAP immunohistological technique described in this paper would provide a very sensitive tool for predicting the dose of antibody carrier to be administered and monitoring the ability of Ber-H2 immunoconjugates to target tumour cells.

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